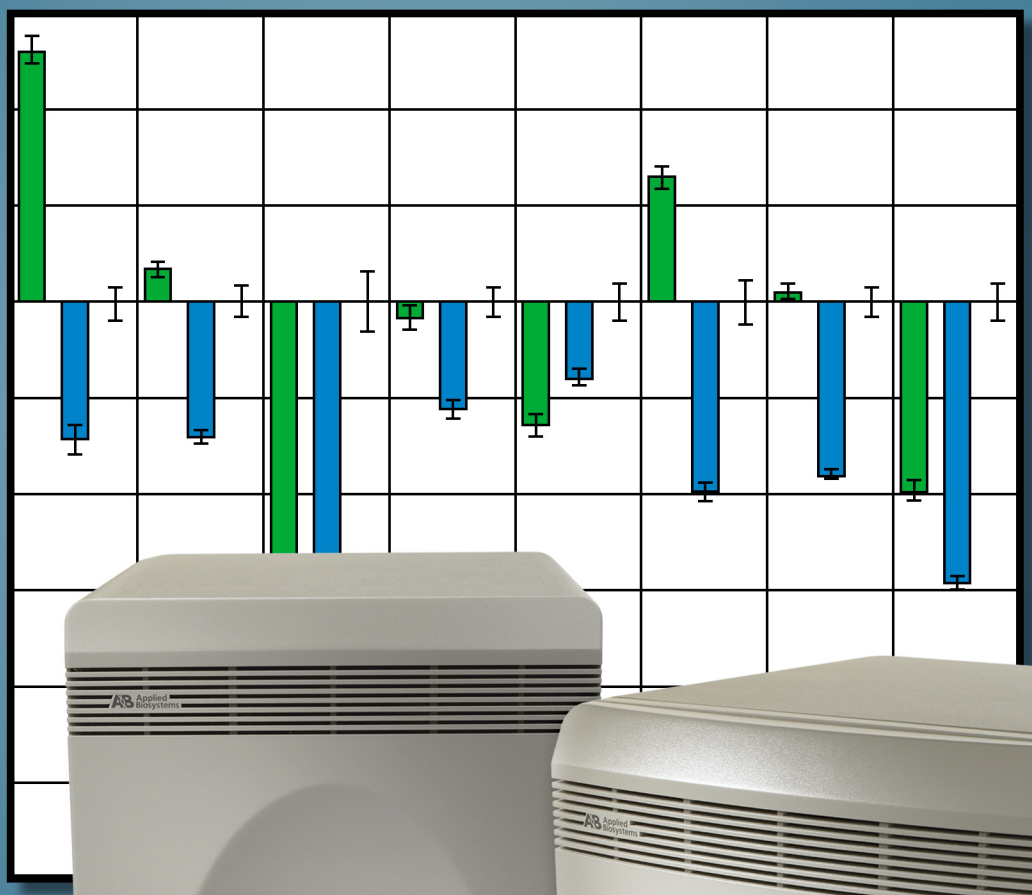
Getting Started Guide

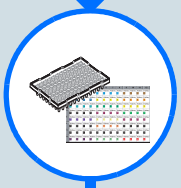
Applied Biosystems

Relative Quantification

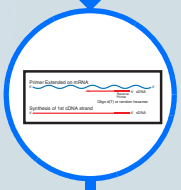
Applied Biosystems 7300/7500 Real Time PCR System



Introduction and Example RQ Experiment



Designing an RQ Experiment



Performing Reverse Transcription

Generating Data from RQ Plates

Generating Data in an RQ Study

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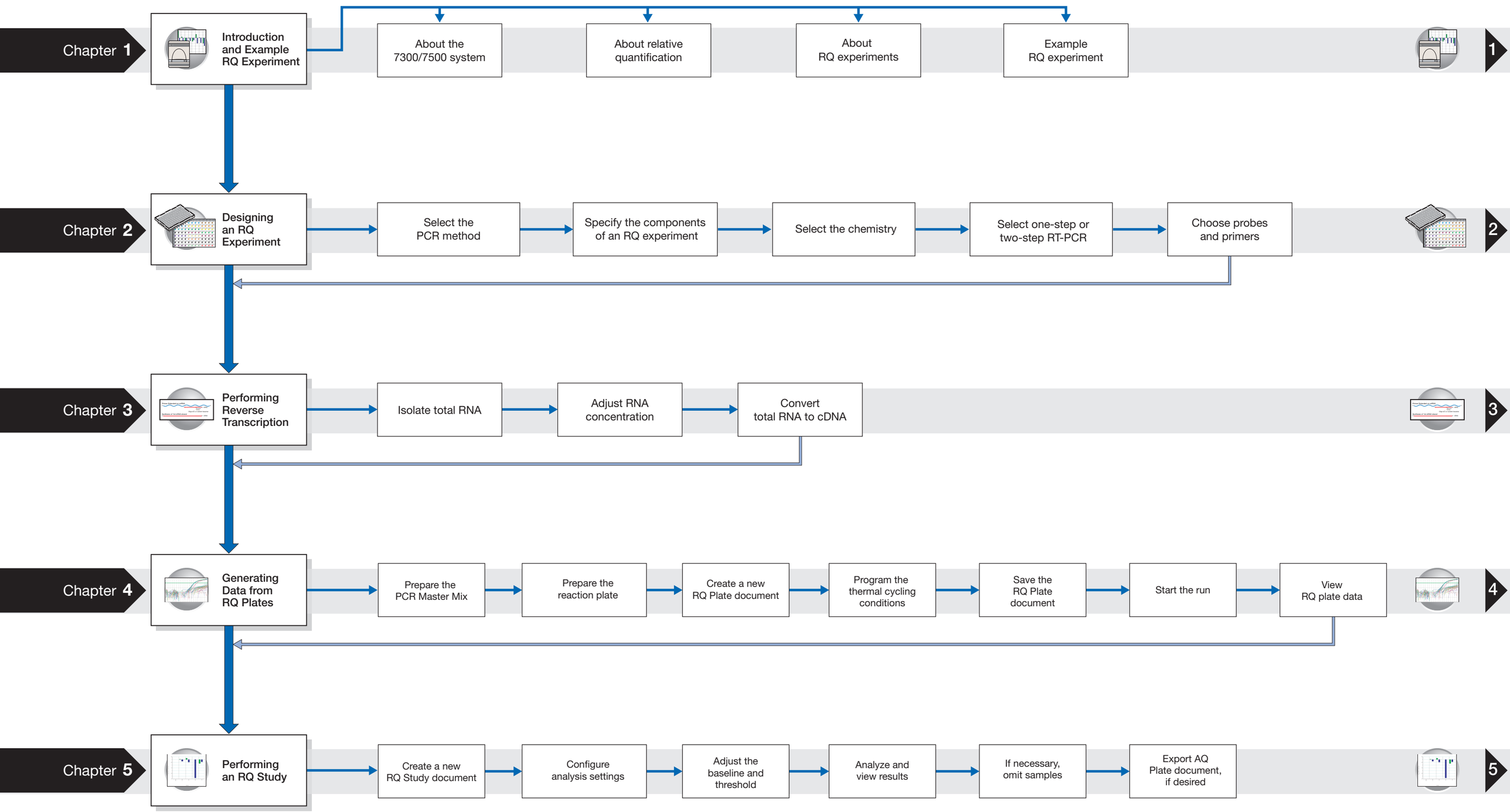
1/2004

RQ Experiment Workflow



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Preface

How to Use This Guide

Purpose of This Guide

This manual is written for principal investigators and laboratory staff who conduct relative quantification studies for gene expression using the Applied Biosystems 7300/7500 Real Time PCR System (7300/7500 system).

Assumptions

This guide assumes that you have:

- Familiarity with Microsoft® Windows® XP operating system.
- Knowledge of general techniques for handling DNA and RNA samples and preparing them for PCR.
- A general understanding of hard drives and data storage, file transfers, and copying and pasting.

If you want to integrate the 7300/7500 system into your existing laboratory data flow system, you need networking experience.

Text Conventions

- **Bold** indicates user action. For example:

  Type **0**, then press **Enter** for each of the remaining fields.
- *Italic* text indicates new or important words and is also used for emphasis. For example:

  Before analyzing, *always* prepare fresh matrix.
- A right arrow bracket (>) separates successive commands you select from a drop-down or shortcut menu. For example:

  Select **File > Open > Spot Set**.

User Attention Words

The following user attention words appear in Applied Biosystems user documentation. Each word implies a particular level of observation or action as described below:

Note: Provides information that may be of interest or help but is not critical to the use of the product.

IMPORTANT! Provides information that is necessary for proper instrument operation, accurate chemistry kit use, or safe use of a chemical.

Indicates a potentially hazardous situation that, if not avoided, may result in minor or moderate injury. It may also be used to alert against unsafe practices.

Indicates a potentially hazardous situation that, if not avoided, could result in death or serious injury.

Safety Refer to the *Applied Biosystems 7300/7500 Real Time PCR System Installation and Maintenance Getting Started Guide* and the *Applied Biosystems* 7300/7500 *Real Time PCR System Site Preparation Guide* for important safety information.

How to Obtain More Information

For more information about using the 7300/7500 system, refer to:

- *Applied Biosystems 7300/7500 Real Time PCR System Online Help*
- *Applied Biosystems 7300/7500 Real Time PCR System Allelic Discrimination Getting Started Guide* (PN 4347822)
- *Applied Biosystems 7300/7500 Real Time PCR System Plus/Minus Getting Started Guide* (PN 4347821)
- *Applied Biosystems 7300/7500 Real Time PCR System Absolute Quantification Getting Started Guide* (PN 4347825)
- *Applied Biosystems 7300/7500 Real Time PCR System Installation and Maintenance Getting Started Guide* (PN 4347828)
- *Applied Biosystems 7300/7500 Real Time PCR System Site Preparation Guide* (PN 4347823)
- *Sequence Detection Systems Chemistry Guide* (PN 4348358)
- *ABI PRISM® 7700 Sequence Detection System User Bulletin #2: Relative Quantitation of Gene Expression (*PN 4303859)

How to Obtain Services and Support

For the latest services and support information for all locations, go to **http://www.appliedbiosystems.com**, then click the link for **Support**.

At the Support page, you can:

- Search through frequently asked questions (FAQs)
- Submit a question directly to Technical Support
- Order Applied Biosystems user documents, MSDSs, certificates of analysis, and other related documents
- Download PDF documents
- Obtain information about customer training
- Download software updates and patches

In addition, the Support page provides access to worldwide telephone and fax numbers to contact Applied Biosystems Technical Support and Sales facilities.

Send Us Your Comments

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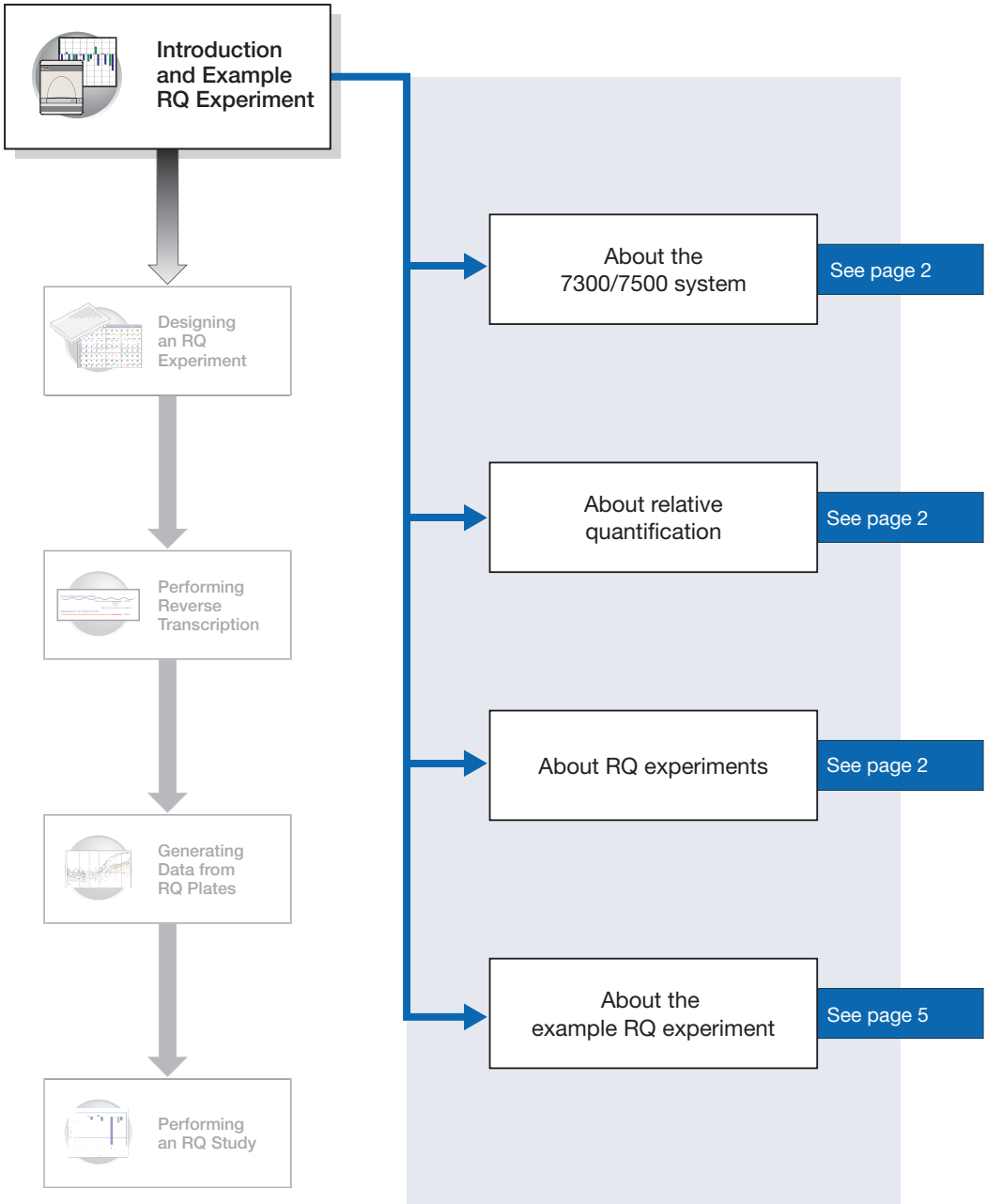
techpubs@appliedbiosystems.com

Chapter 1

Introduction and Example RQ Experiment

1

Overview



Notes

About the 7300/7500 System

Description

The Applied Biosystems 7300/7500 Real Time PCR System (7300/7500 system) uses fluorescent-based PCR chemistries to provide quantitative detection of nucleic acid sequences using real-time analysis and qualitative detection of nucleic acid sequences using end-point and dissociation-curve analysis.

Relative Quantification Assay

The 7300/7500 system allows you to perform several assay types using plates or tubes in the 96-well format. This guide describes the relative quantification (RQ) assay type.

For information about the other assay types, refer to the *Sequence Detection Systems Chemistry Guide (SDS Chemistry Guide)* and the Online Help for the 7300/7500 system (Online Help).

About Relative Quantification

Definition

Relative quantification determines the change in expression of a nucleic acid sequence (target) in a test sample relative to the same sequence in a calibrator sample. The calibrator sample can be an untreated control or a sample at time zero in a time-course study (Livak and Schmittgen, 2001). For example, relative quantification is commonly used to compare expression levels of wild-type with mutated alleles or the expression levels of a gene in different tissues.

RQ provides accurate comparison between the initial level of template in each sample, without requiring the exact copy number of the template. Further, the relative levels of templates in samples can be determined without the use of standard curves.

Real-time PCR Assays

RQ is performed using real-time PCR. In real-time PCR assays, you monitor the progress of the PCR as it occurs. Data are collected throughout the PCR process rather than at the end of the PCR process (end-point PCR).

In real-time PCR, reactions are characterized by the point in time during cycling when amplification of a target is first detected rather than by the amount of target accumulated at the end of PCR.

There are two types of quantitative real-time PCR: absolute and relative.

About RQ Experiments

RQ Experiment Workflow

In this document, the term “RQ experiment” refers to the entire process of relative quantification, beginning with generating cDNA from RNA (reverse transcription) and ending with analyzing an RQ study. The RQ experiment workflow is shown on page iii.

Notes

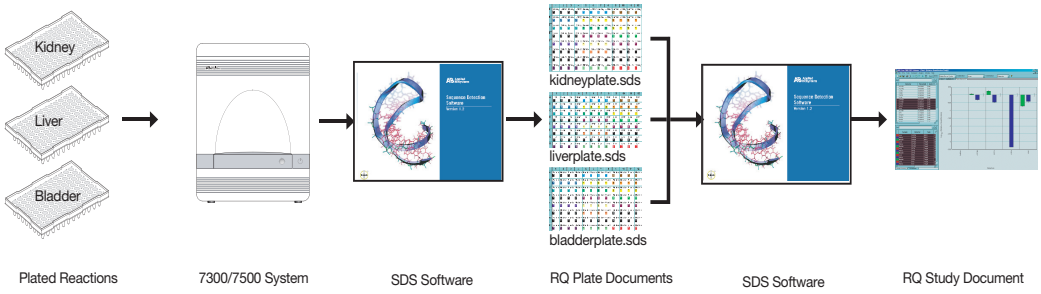
RQ Studies with the 7300/7500 System

The data-collection part of an RQ assay is a single-plate document, called the RQ Plate. Amplification data from PCR runs is stored with sample setup information on the plate.

The data-analysis part of an RQ assay is a multi-plate document, called the RQ Study. You can analyze up to ten RQ plates in a study. RQ Study documents neither control the instrument, nor do they provide tools for setting up or modifying plates.

IMPORTANT! RQ Study software is an optional package for the 7300 instrument but is standard for the 7500 instrument.

The following figure illustrates the RQ Study process.



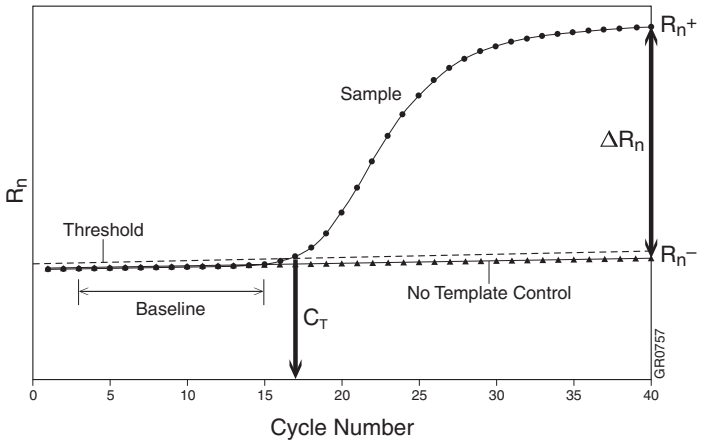
Note: The 7300/7500 system software uses only the comparative method ($\Delta\Delta C_T$) to calculate relative quantities of a nucleic acid sequence.

Terms Used in Quantification Analysis

Term	Definition
Baseline	The initial cycles of PCR in which there is little change in fluorescence signal.
Threshold	A level of ΔR_n—automatically determined by the SDS software or manually set—used for C_T determination in real-time assays. The level is set to be above the baseline and sufficiently low to be within the exponential growth region of the amplification curve. The threshold is the line whose intersection with the Amplification plot defines the C_T.
Threshold cycle (C_T)	The fractional cycle number at which the fluorescence passes the threshold.
Passive reference	A dye that provides an internal fluorescence reference to which the reporter dye signal can be normalized during data analysis. Normalization is necessary to correct for fluorescence fluctuations caused by changes in concentration or in volume.
Reporter dye	The dye attached to the 5′ end of a TaqMan probe. The dye provides a signal that is an indicator of specific amplification.
Normalized reporter (R_n)	The ratio of the fluorescence emission intensity of the reporter dye to the fluorescence emission intensity of the passive reference dye.
Delta R_n (ΔR_n)	The magnitude of the signal generated by the specified set of PCR conditions. ($\Delta R_n = R_n$ – baseline)

Notes

The figure below shows a representative amplification plot and includes some of the terms defined in the previous table.



Required User-Supplied Materials

Item	Source
ABI PRISM™ 6100 Nucleic Acid PrepStation	Applied Biosystems (PN 6100-01)
High Capacity cDNA Archive Kit	Applied Biosystems (PN 4322171)
TaqMan® Universal PCR Master Mix	Applied Biosystems (PN 4304437)
MicroAmp® Optical 96-Well Reaction Plate	Applied Biosystems (PN 4306757)
Optical Adhesive Cover	Applied Biosystems (PN 4311971)
Labeled primers and probes from one of the following sources: • Assays-on-Demand™ Gene Expression Products (predesigned primers and probes) • Assays-by-Design™ service (predesigned primers and probes) • Primer Express Software (custom-designed primers and probes)	 • Applied Biosystems Web site • Contact your Applied Biosystems Sales Representative • PN 4330710 (1-user license) PN 4330709 (10-user license) PN 4330708 (50-user license)
Reagent tubes with caps, 10-mL	Applied Biosystems (PN 4305932)
Centrifuge with adapter for 96-well plates	Major laboratory supplier (MLS)
Gloves	MLS
Microcentrifuge	MLS
Microcentrifuge tubes, sterile 1.5-mL	MLS

Notes

Item	Source
Nuclease-free water	MLS
Pipette tips, with filter plugs	MLS
Pipettors, positive-displacement	MLS
Safety goggles	MLS
Vortexer	MLS

Example RQ Experiment

Overview

To better illustrate how to design, perform, and analyze RQ experiments, this section guides you through an example experiment. The example experiment represents a typical RQ experiment setup that you can use as a quick-start procedure to familiarize yourself with the RQ workflow. Detailed steps in the RQ workflow are described in the subsequent chapters of this guide. Also in the subsequent chapters are Example Experiment boxes that provide details for some of the related steps in the example experiment.

Description

The objective of the example RQ experiment is to compare the levels of expression of 23 genes in the liver, kidney, and bladder tissue of an individual.

The experiment is designed for singleplex PCR – samples and endogenous controls were amplified in separate wells. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) serves as the endogenous control. Four replicates of each sample and endogenous control are amplified. (In this experiment, an entire 96-well-plate is devoted to each tissue because the four replicates of each of the 23 genes plus the endogenous control require all 96 wells.)

Predesigned and labeled primer/probe sets are selected from the Applied Biosystems Assays-on-Demand™ product line.

Reactions are set up for two-step RT-PCR, where the High Capacity cDNA Archive Kit and the TaqMan® Universal PCR Master Mix are used for reverse transcription and PCR, respectively.

Data are generated by running three RQ plates, one for each tissue.

All three plates are analyzed in an RQ study, with the liver samples serving as the calibrator.

Notes

Example RQ Experiment Procedure

1. Design the experiment, as explained in Chapter 2.
   a. Designate the targets, calibrator, endogenous control, and replicates.
   b. Order the reagents for TaqMan® probe-based chemistry.
   c. Order the appropriate Assays-on-Demand™ products, which provide predesigned primers and probes for the 23 genes.
2. Isolate total RNA from liver, kidney, and bladder tissue, as explained in Chapter 3.
3. Generate cDNA from total RNA using the High Capacity cDNA Archive Kit.
   a. Prepare the reverse transcription (RT) master mix as indicated in the table to the right.

      Additional guidelines are provided in the *High Capacity cDNA Archive Kit Protocol*.

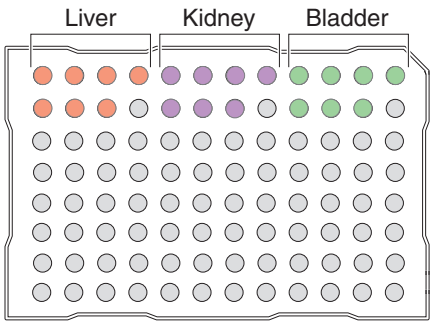
WARNING **CHEMICAL HAZARD.** **10 × RT Buffer** may cause eye, skin, and respiratory tract irritation. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

RT Master Mix		
Component	**µL/Reaction**	**µL/21 reactions[a]**
10× Reverse Transcription Buffer	10	210
25× dNTPs	4	84
10× random primers	10	210
MultiScribe™ Reverse Transcriptase, 50 U/µL	5	105
Nuclease-free water	21	441
Total	50	1050

a. Each RT reaction is 100 µL (see step 3b). If you need 5 µL cDNA for each of 104 PCR reactions per tissue (see step 4), you need 6 RT reactions per tissue. Extra volume (enough for one additional RT reaction per tissue) is included to account for pipetting losses, as well as extra cDNA for archiving.

   b. Prepare the cDNA archive plate by pipetting into each well of the plate:
      - 50 µL RT master mix
      - 30 µL nuclease-free water
      - 20 µL RNA sample

      Make sure the amount of total RNA converted to cDNA is 10 to 100 ng in 5 µL for each 50-µL PCR reaction.



Notes

c. Program the thermal cycler using the indicated parameter values for the RT step of the two-step RT-PCR method.

Note: You have the option to use one-step RT-PCR, as explained in "Selecting One- or Two-Step RT-PCR" on page 16.

Step Type	Time	Temperature
HOLD	10 min	25 °C
HOLD	120 min	37 °C

d. Store the cDNA at –20 °C until use.

4. Prepare the PCR master mix as indicated in the table to the right.

   See Chapter 4 for more information.

Note: The reaction volumes for Assay-by-Design products are specified in the product insert; those for primers and probes designed with Primer Express software follow the universal assay conditions described in Chapter 4.

CAUTION CHEMICAL HAZARD. **TaqMan Universal PCR Master Mix** may cause eye and skin irritation. Exposure may cause discomfort if swallowed or inhaled. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

PCR Master Mix			
Reaction Component	**µL/ Sample**	**µL/ 5 Reactions[b]**	**Final Concen-tration**
TaqMan Universal PCR Master Mix (2×)	25.0	125.0	1×
20× Assays-on-Demand™ Gene Expression Assay Mix[a]	2.5	12.5	1×
cDNA sample	5.0	25.0	10 to 100 ng
Nuclease-free water	17.5	87.5	—
Total	50.0	250	—

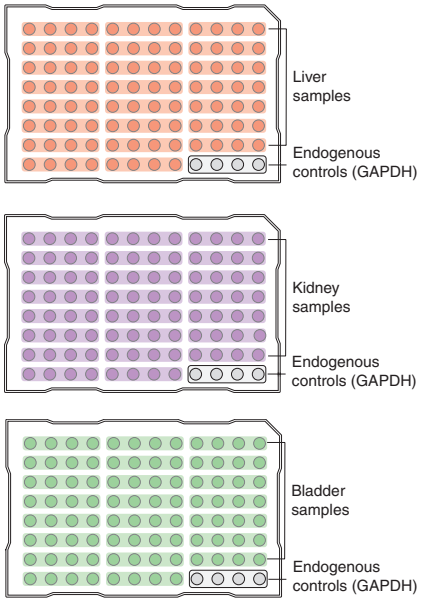
a. Contains forward and reverse primers and labeled probe.
b. 24 master mixes are prepared, one for each of 23 genes plus the endogenous control. Volume for five reactions (4 replicates plus extra) to account for pipetting losses.

5. Prepare the reaction plates.

   a. Label the reaction plates, ensuring that you include an endogenous control on each plate.

   b. Pipette 50 µL of the appropriate PCR master mix (containing cDNA) into each well of the plate.

   c. Keep the reaction plates on ice until you are ready to load them into the 7300/7500 system.



Notes

6. Create an RQ Plate document as described in "Creating a Relative Quantification (RQ) Plate Document" on page 26. Briefly,

   a. Select **File > New**.

   b. Select **Relative Quantification (ddCt) Plate** in the Assay drop-down list, then click **Next >**.

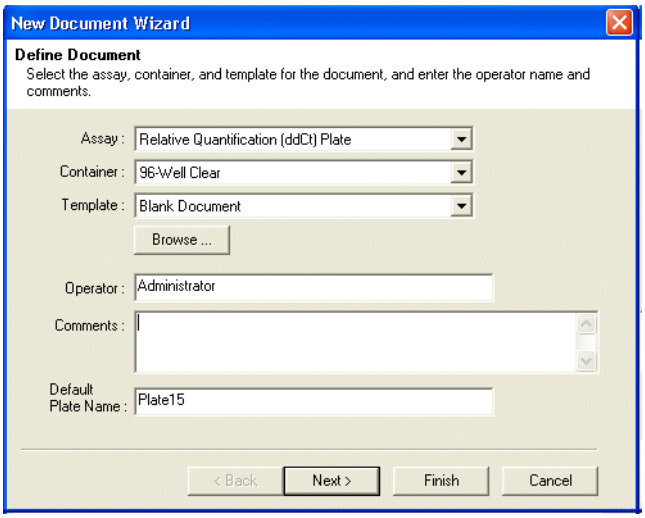
   IMPORTANT! You cannot use AQ Plate documents for RQ assays and vice versa. The information stored in AQ and RQ Plate documents is not interchangeable.

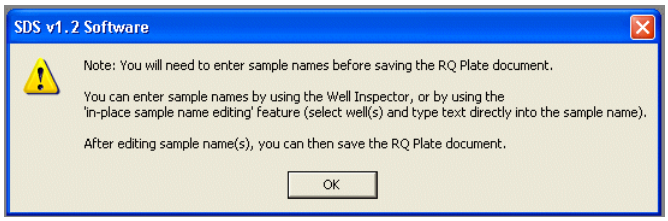
   c. Add detectors to the plate document, then click **Next >**.

   d. Specify the detectors and tasks for each well, then click **Finish**.

   You cannot add RQ plates to RQ studies unless you have specified sample names, as indicated in the message shown to the right. Click **OK**.

   The SDS software displays the Well Inspector.

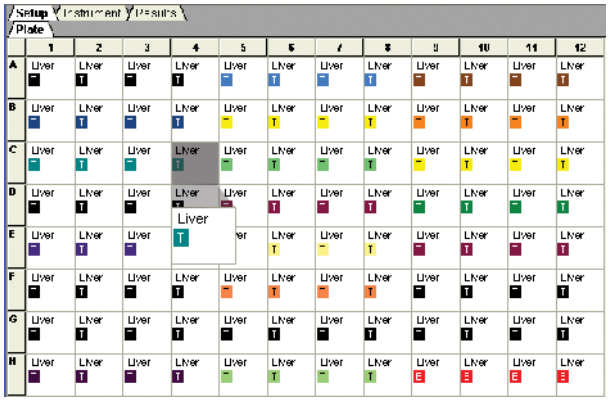



SDS v1.2 Software
Note: You will need to enter sample names before saving the RQ Plate document.
You can enter sample names by using the Well Inspector, or by using the 'in-place sample name editing' feature (select well(s) and type text directly into the sample name).
After editing sample name(s), you can then save the RQ Plate document.
OK

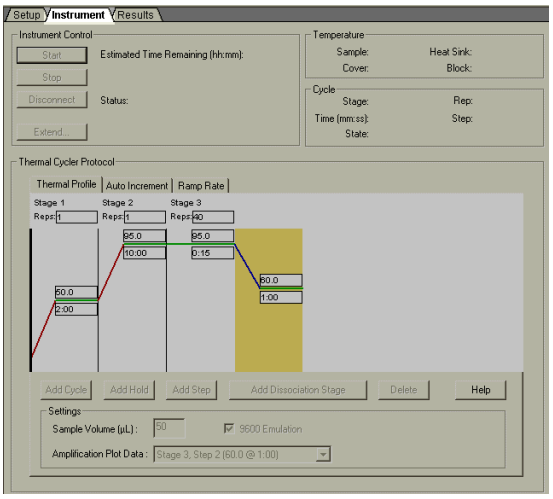
7. Enter the sample names in the Well Inspector (**View > Well Inspector**).

   IMPORTANT! If your experiment does not use all the wells on a plate, do not omit the wells from use at this point. You can omit unused wells after the run is completed. For more information about omitting wells, refer to the Online Help.

   The figure on the right shows a completed plate set up.



Notes



8. Start the RQ run.

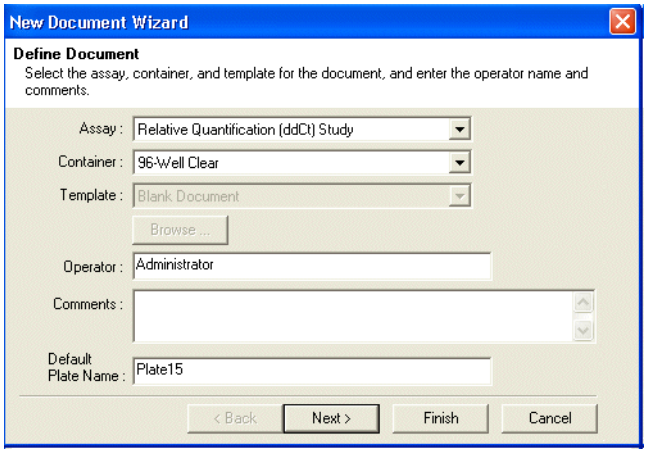
   a. Select the **Instrument** tab. By default, the standard PCR conditions for the PCR step of the two-step RT-PCR method are displayed.

   b. Select **File > Save As**, enter a name for the RQ Plate document, then click **Save**.

   c. Load the plate into the instrument.

   d. Click **Start**.

   After the run, a message indicates if the run is successful or if errors were encountered.



9. Create an RQ Study document as described in "Creating an RQ Study Document" on page 36. Briefly,

   a. Select **File > New**.

   b. Select **Relative Quantification (ddCt) Study** in the Assay drop-down list, then click **Next >**.

   IMPORTANT! RQ Studies are an optional add-on for the 7300 instrument; they are built-in for the 7500 instrument.

   c. Click **Add** to add plates to the study, then click **Open**.

   Note: You can add up to 10 RQ plates to an RQ study.

   d. Click **Finish**.

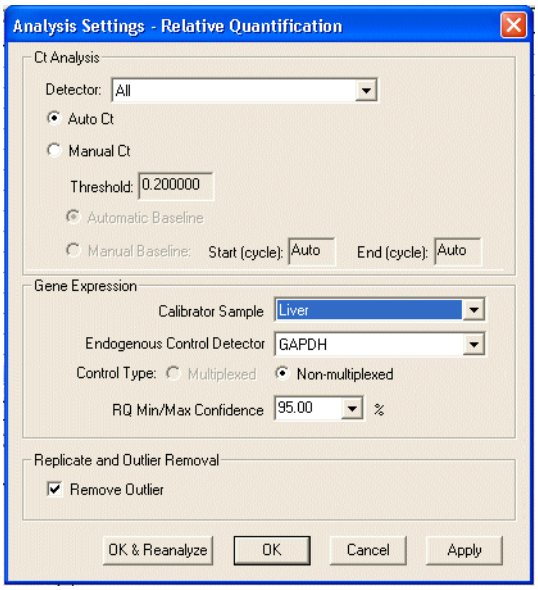
Notes

10. Analyze the RQ data, as explained in Chapter 5.

a. Configure analysis settings ( ), using the Auto Ct option and analyze the data.

Note: See "Configuring Analysis Settings" on page 38 for details.

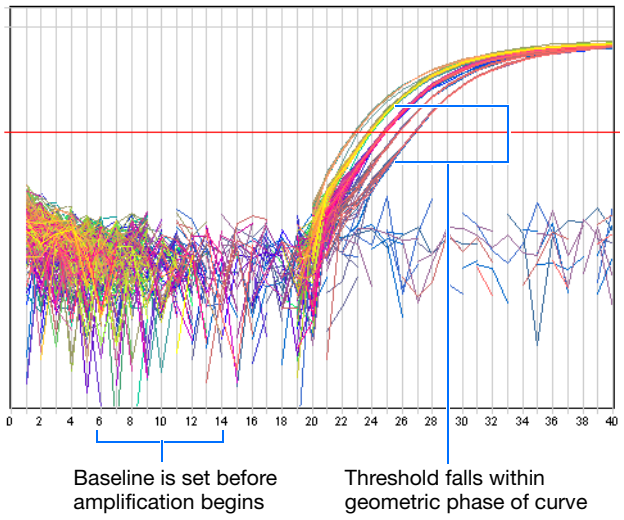
If you know the optimal baseline and threshold settings for your experiment, you can use the Manual Ct and Manual Baseline options.



b. If necessary, manually adjust the baseline and threshold.

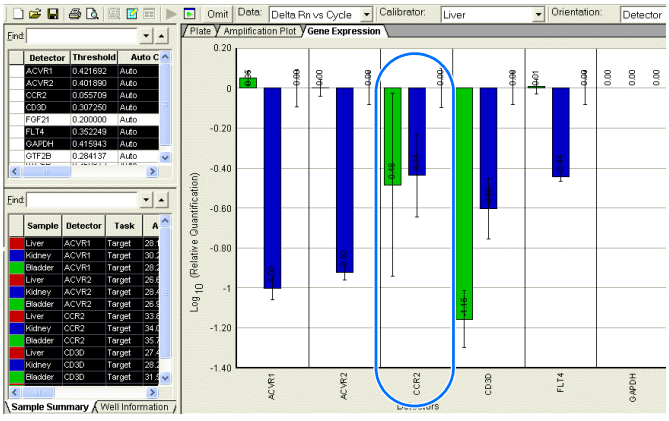
Note: See "Adjusting the Baseline and Threshold" on page 40.

c. Click , or select **Analysis > Analyze** to reanalyze the data.


0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40
Baseline is set before amplification begins
Threshold falls within geometric phase of curve

d. View analysis results by clicking a tab in the RQ Results pane.

e. If desired, save the RQ Study document.



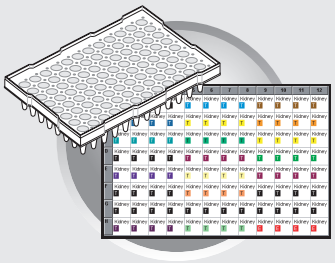
Conclusion

As shown in the figure on the right, expression levels of CCR2 are greater in the liver than in the kidney or bladder tissues of this individual.

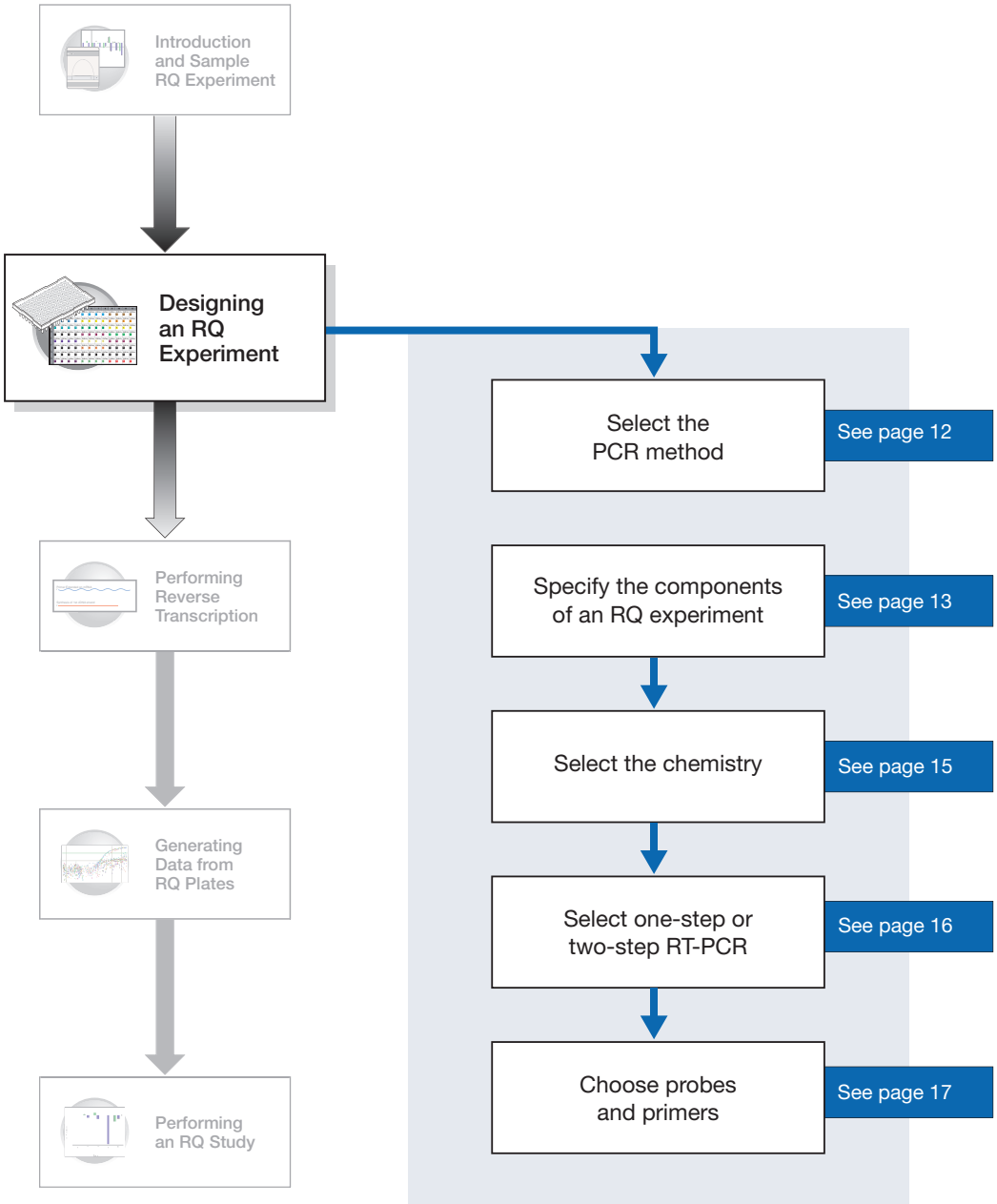
Notes

Chapter 2

Designing an RQ Experiment



Workflow



Notes

Selecting the PCR Method

Types of PCR Methods

PCR is performed as either of the following:

- A singleplex reaction, where a single primer pair is present in the reaction tube or well. Only one target sequence or endogenous control can be amplified per reaction.
- A multiplex reaction, where two or more primer pairs are present in the reaction. Each primer pair amplifies either a target sequence or an endogenous control.

Selection Criteria

Both methods give equivalent results for relative quantification experiments. To select a method, consider the:

- Type of chemistry you use to detect PCR products – Singleplex PCR can use either SYBR® Green or TaqMan reagent-based chemistry. Multiplex PCR can use only TaqMan chemistry.
- Amount of time you want to spend optimizing and validating your experiment – Amplifying target sequences and endogenous controls in separate reactions (singleplex PCR) requires less optimization and validation than multiplex PCR.

  Among the factors to consider in multiplex PCR are primer limitation, the relative abundance of the target and reference sequences (the endogenous control must be more abundant than the targets), and the number of targets in the study.

  IMPORTANT! As the number of gene targets increases, the singleplex format is typically more effective than the multiplex format because less optimization is required.

Additionally, running multiple reactions in the same tube multiplex PCR increases throughput and reduces the effects of pipetting errors.

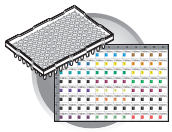
For more information about multiplex and singleplex PCR, refer to the *SDS Chemistry Guide* (PN 4348358).

Example Experiment

The singleplex PCR method is used in the example experiment because:

- The number of targets to be amplified (23 genes, plus one endogenous control) is large
- Optimization and validation requirements are reduced for singleplex experiments

Notes



Specifying the Components of an RQ Experiment

After you decide to use the singleplex or multiplex method, you need to specify the required components of the RQ experiment for every sample:

- A target – The nucleic acid sequence that you are studying.
- A calibrator – The sample used as the basis for comparative results.
- An endogenous control – A gene present at a consistent expression level in all experimental samples. By using an endogenous control as an active reference, you can normalize quantification of a cDNA target for differences in the amount of cDNA added to each reaction. Note that:
  - Each sample type (for example, each tissue in a study comparing multiple tissues) requires an endogenous control.
  - If samples are spread across multiple plates, each plate must have an endogenous control. Additionally, every plate must include an endogenous control for every sample type on the plate.

  Typically, housekeeping genes such as β-actin, glyceraldehyde-3-phosphate (GAPDH), and ribosomal RNA (rRNA), are used as endogenous controls, because their expression levels tend to be relatively stable.
- Replicate wells – For relative quantification studies, Applied Biosystems recommends the use of three or more replicate reactions per sample and endogenous control to ensure statistical significance.

For more information about these requirements, refer to the *SDS Chemistry Guide*.

Notes

Example Experiment

In the example experiment, the objective is to compare the expression levels of several genes in the liver, kidney, and bladder tissue of an individual. The 23 genes of interest, including ACVR1, ACVR2, CCR2, CD3D, and FLT4, are the targets and the liver samples serve as the calibrator.

The SDS software sets gene expression levels for the calibrator samples to 1. Consequently, if more ACRV1 is in the kidney than in the liver, the gene expression level of ACRV1 in the kidney is greater than 1. Similarly, if less CD3D is in the bladder than in the liver, the gene expression level of CD3D in the bladder is less than 1.

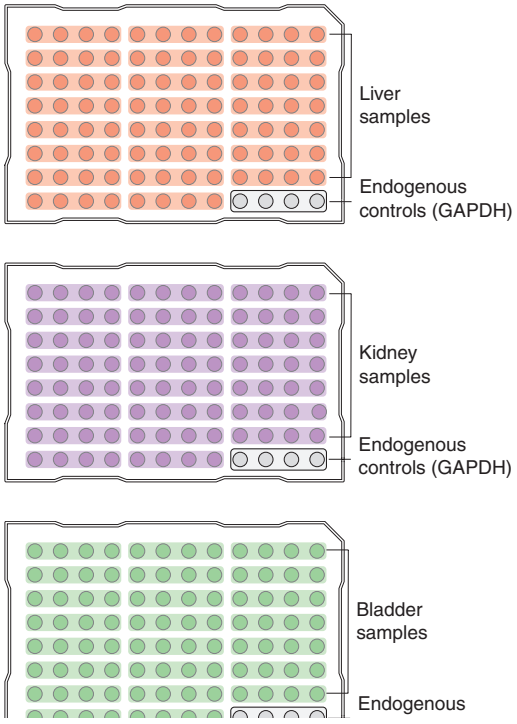
Because RQ is based on PCR, the more template in a reaction, the more the PCR product and the greater the fluorescence. To adjust for possible differences in the amount of template added to the reaction, GAPDH serves as an endogenous control. (Expression levels of the endogenous control are subtracted from expression levels of target genes.) An endogenous control is prepared for each tissue.

The experiment includes three sets of endogenous controls—one for each tissue. Also, the endogenous control for each tissue must be amplified on the same plate as the target sequences for that tissue. Finally, note that the experiment uses the singleplex PCR format, and therefore, the endogenous controls are amplified in wells different from the target wells.

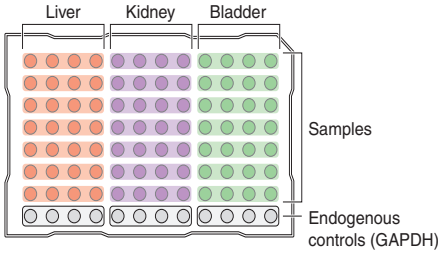
Four replicates of each sample and endogenous control are performed to ensure statistical significance (see below).

Note: The example RQ experiment requires a separate plate for each of the three tissues because of the large number of genes being studied. Experiments can also be designed so that several samples are amplified on the same plate, as shown in the following table.

In the example RQ experiment, each plate contains a single sample type (tissue). The endogenous control for each tissue is on the same plate as the targets for that tissue.



If the example experiment were run with multiple sample types on the same plate, an endogenous control for each sample type must also be included on the same plate, as shown here.



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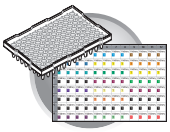
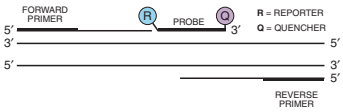
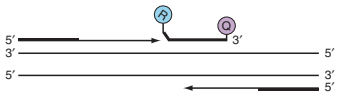
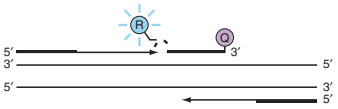
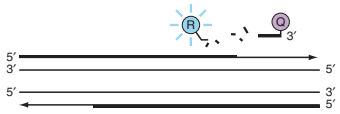
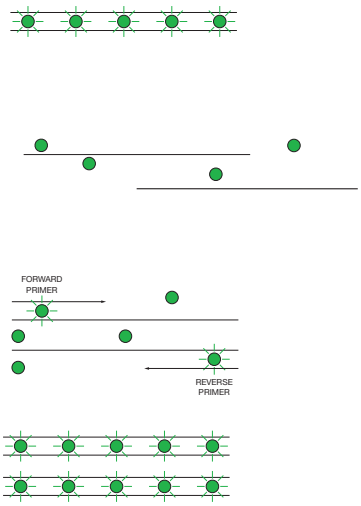


Selecting the Chemistry

About Chemistries

Applied Biosystems offers two types of chemistries that you can use to detect PCR products on real-time instruments, as explained in the following table. Both TaqMan probe-based and SYBR Green I dye chemistries can be used for either one- or two-step RT-PCR. For more information about these chemistries, refer to the *SDS Chemistry Guide*.

Chemistry	Process
TaqMan® reagents or kits **Description** TaqMan reagent-based chemistry uses a fluorogenic probe to enable detection of a specific PCR product as it accumulates during PCR cycles. **Advantages** • Increases specificity with a probe. Specific hybridization between probe and target generates fluorescence signal. • Provides multiplex capability • Optimized assays available • Allows 5′-nuclease assay to be carried out during PCR	Polymerization  Step 1: A reporter (R) and a quencher (Q) are attached to the 5' and 3' ends of a TaqMan probe. Strand Displacement  Step 1 continued: when both dyes are attached to the probe, reporter dye emission is quenched. Cleavage  Step 2: During each extension cycle, the AmpliTaq Gold® DNA polymerase cleaves the reporter dye from the probe. Polymerization Completed  Step 3: After being separated from the quencher, the reporter dye emits its characteristic fluorescence.
SYBR® Green I reagents **Description** Uses SYBR Green I dye, a double-stranded DNA binding dye, to detect PCR products as they accumulate during PCR cycles. **Advantages** • Reduces cost (no probe needed) • Amplifies all double-stranded DNA • Yields a melting profile of distinct PCR runs • Increases sensitivity for detecting amplification products relative to product length **Limitations** Binds nonspecifically to all double-stranded DNA sequences. To avoid false positive signals, check for nonspecific product formation using dissociation curve or gel analysis.	 Step 1: Reaction setup The SYBR® Green I dye fluoresces when bound to double-stranded DNA. Step 2: Denaturation When the DNA is denatured, the SYBR® Green I dye is released and the fluorescence is drastically reduced. Step 3: Polymerization During extension, primers anneal and PCR product is generated. Step 4: Polymerization completed SYBR® Green I dye binds to the double-stranded product, resulting in a net increase in fluorescence detected by the instrument.

Notes

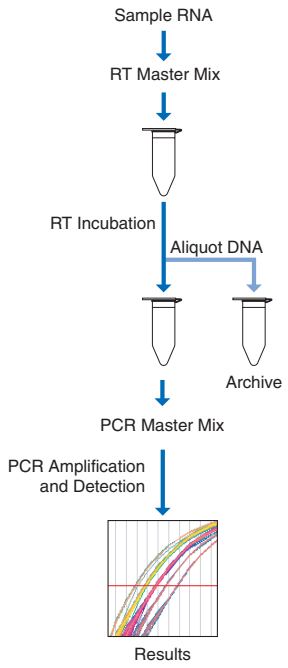
Selecting One- or Two-Step RT-PCR

When performing real-time PCR, you have the option of performing reverse transcription (RT) and PCR in a single reaction (one-step) or in separate reactions (two-step).The reagent configuration you use depends on whether you are performing one-step or two-step RT-PCR:

- Two-step RT-PCR is performed in two separate reactions: first, total RNA is reverse transcribed into cDNA, then the cDNA is amplified by PCR. This method is useful for detecting multiple transcripts from a single cDNA template or for storing cDNA aliquots for later use. AmpErase® UNG enzyme can be used to prevent carryover contamination.

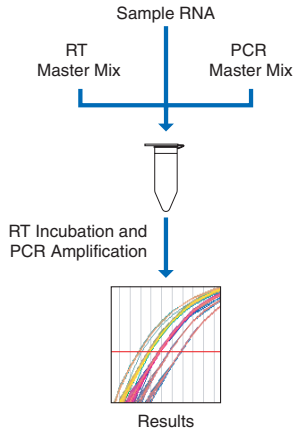
  IMPORTANT! This guide assumes that RQ experiments are designed using two-step RT-PCR. For additional options, refer to the *SDS Chemistry Guide*.

Two-Step RT-PCR



- In one-step RT-PCR, RT and PCR take place in one buffer system, which provides the convenience of a single-tube preparation for RT and PCR amplification. However, you cannot use the carryover prevention enzyme, AmpErase® UNG (uracil-N-glycosylase), with one-step RT-PCR. For more information about UNG, refer to the *SDS Chemistry Guide*.

One-Step RT-PCR



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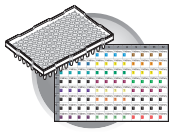


Recommended Kits for Two-Step RT-PCR			
Chemistry	**Step**	**Reagent**	**Part Number**
TaqMan reagents or kits	RT	High Capacity cDNA Archive Kit	4322171
	PCR	TaqMan Universal PCR Master Mix	4304437
SYBR Green I reagents or kits	RT	High Capacity cDNA Archive Kit	4322171
	PCR	SYBR Green Master Mix	4309155
	RT and PCR	SYBR Green RT-PCR Reagents	4310179

Example Experiment

Premade probes and primers for all the genes of interest are available from the Assays-on-Demand™ product line, which uses TaqMan chemistry. Two-step RT-PCR is performed using the reagents recommended for TaqMan reagent- or kit-based chemistry in the table above.

Choosing the Probes and Primers

Choose probe and primer sets for both your target and endogenous control sequences. Applied Biosystems provides three options for choosing primers and probes:

- Assays-on-Demand™ Gene Expression Products – Provide you with optimized, ready-to-use TaqMan 5′-nuclease assays for human, mouse, or rat transcripts. For information on available primer/probe sets, go to:

  http://www.allgenes.com
- Assays-by-Design℠ Service – Designs, synthesizes, formulates, and delivers quality-controlled primer and probe sets. Use this service if the assay you need is not currently available. To place an order, contact your Applied Biosystems representative.
- Primer Express® Software – Helps you design primers and probes for your own quantification assays. For more information about using this software, refer to the *Primer Express Software v2.0 User's Manual* (PN 4329500).

  Applied Biosystems provides assay design guidelines that have been developed specifically for quantification assays. When followed, these guidelines provide a reliable system for assay design and optimization. For information about the assay design guidelines, refer to the *SDS Chemistry Guide*.

If you ordered Assays-on-Demand or Assays-by-Design products, probes are already labeled with a reporter dye. If you design your own assays, you need to specify a reporter dye for your custom probe(s). For singleplex experiments, you can use the same dye for targets and endogenous control(s). For multiplex experiments, the probe for the target is typically labeled with FAM dye and that for the endogenous control with VIC® dye.

Notes

Example Experiment

For the example experiment, primers and probes for all the genes being studied are obtained from Applied Biosystems Assays-on-Demand™ products. Each assay consists of two unlabeled PCR primers and a FAM™ dye-labeled TaqMan® MGB probe, provided as a 20× assay mix.

In the example experiment, all target probes are labeled with FAM dye; the endogenous control is also labeled with FAM dye.

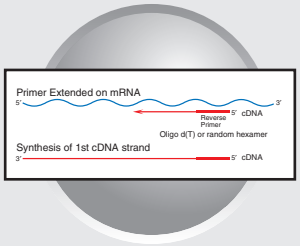
The following table provides the gene symbol, gene name, and Applied Biosystems Assay ID number (provided on the Web site) for five of the genes studied in the example experiment, plus the endogenous control.

Gene Symbol	Gene Name	Assay ID #
ACVR1	acrosomal vesicle protein I	Hs00153836 m1
ACVR2	activin A receptor, type II	Hs00155658_m1
CCR2	chemokine (C-C motif) receptor 2	Hs00174150_m1
CD3D	CD3D antigen, delta polypeptide (TiT3 complex)	Hs00174158_m1
FLT4	fms-related tyrosine kinase 4	Hs00176607 m1
GAPDH	glyceraldehyde-3-phosphate dehydrogenase	Hs99999905 m1

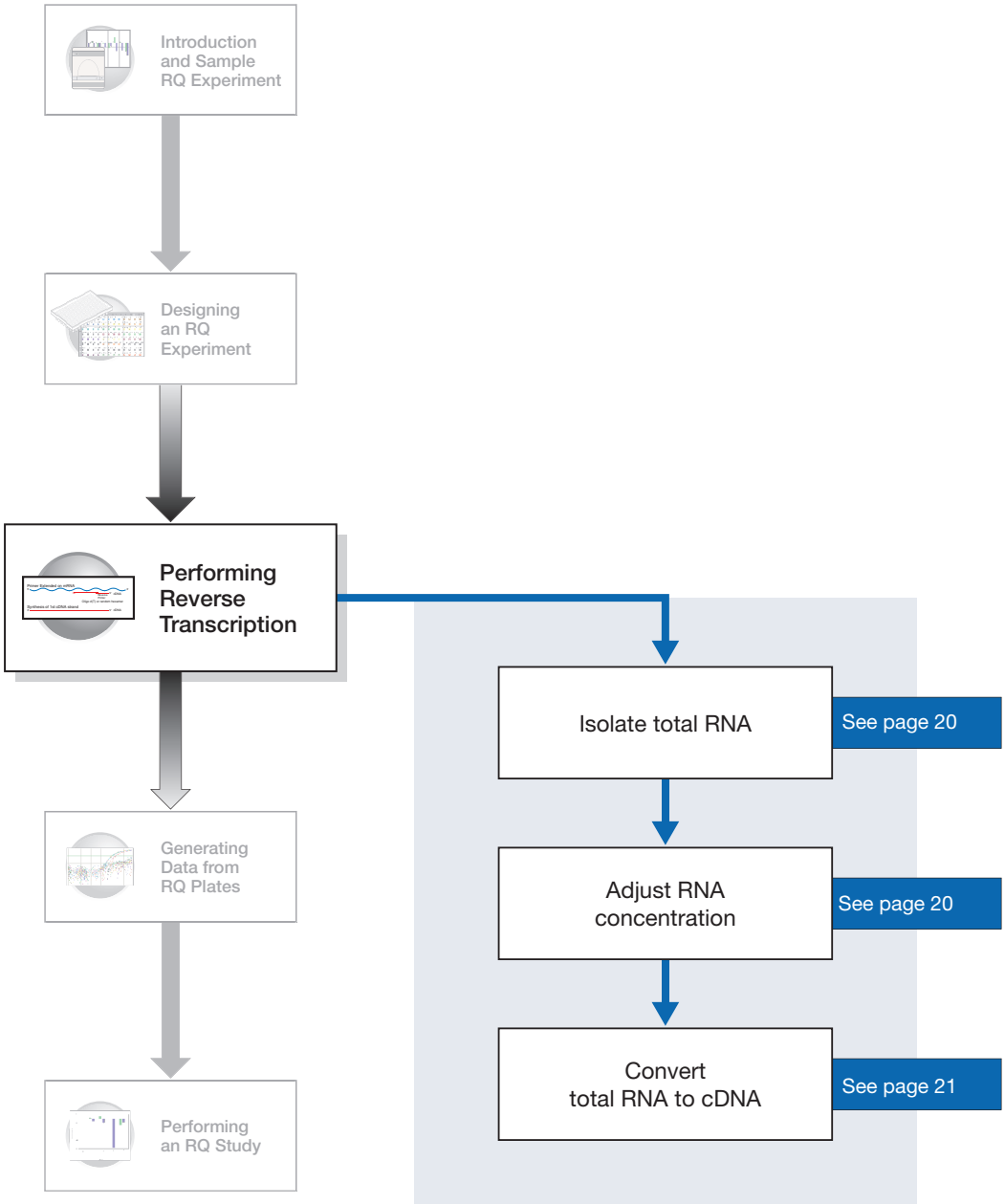
Notes

Chapter 3

Performing Reverse Transcription



Workflow



Notes

Guidelines for Preparing RNA

Isolating RNA

Applied Biosystems supplies several instrument systems and chemistries for RNA isolation from a variety of starting materials, such as blood, tissue, cell cultures, and plant material.

System	Part Number
ABI PRISM™ 6100 Nucleic Acid PrepStation	6100-01
Total RNA Chemistry Reagents:	
Nucleic Acid Purification Elution Solution	4305893
Nucleic Acid Purification Lysis Solution	4305895
Nucleic Acid Purification Wash Solution I	4305891
Nucleic Acid Purification Wash Solution II	4305890
AbsoluteRNA Wash Solution (DNase treatment)	4305545
Tempus™ Blood RNA Tubes (For collection, stabilization, and isolation of total RNA in whole blood for gene analysis using the 6100 PrepStation)	4342972
Isolation of Total RNA from Whole Blood and from Cells Isolated from Whole Blood Protocol	4332809
Tempus™ Blood RNA Tube and Large Volume Consumables Protocol	4345218
Tissue RNA Isolation: Isolation of Total RNA from Plant and Animal Tissue Protocol	4330252

Quality of RNA

The total RNA you use for RQ experiments should:

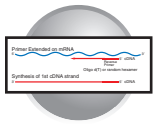
- Have an $A_{260/280}$ greater than 1.9
- Be intact when visualized by gel electrophoresis
- Not contain RT or PCR inhibitors

The *High Capacity cDNA Archive Kit Protocol* (4312169) contains additional guidelines for preparing the RNA template.

Adjusting the Starting Concentration of Total RNA

The High Capacity cDNA Archive Kit is optimized to convert 0.1 to 10 µg of total RNA to cDNA. Convert enough total RNA so that the final concentration of total RNA converted to cDNA is 10 to 100 ng in 5 µL for each 50-µL PCR reaction.

Notes



Converting Total RNA to cDNA

Using the High Capacity cDNA Archive Kit

Use the High Capacity cDNA Archive Kit (PN 4322171) to perform the first step (RT) in the two-step RT-PCR method. Follow the manual method for converting total RNA into cDNA, as specified in the *High Capacity cDNA Archive Kit Protocol* (PN 4322169).

IMPORTANT! The protocol is not shipped with the High Capacity cDNA Archive Kit. Download the protocol from

http://docs.appliedbiosystems.com/search.taf

To search for the document, select **ABI PRISM™ 6100 Nucleic Acid PrepStation** in the Product list box, then click **Search** at the bottom of the page. The protocol is listed under the Protocols heading.

Thermal Profile Parameters for RT

The High Capacity cDNA Archive Kit uses the following thermal profile parameters for the RT step.

Step Type	Time	Temperature
HOLD	10 min	25 °C
HOLD	120 min	37 °C

Note: Thermal cycling conditions for one-step RT-PCR are described on page 30.

Notes

Storing cDNA

After cDNA conversion, store all cDNA samples at –15 to –25 °C. To minimize repeated freeze-thaw cycles of cDNA, store cDNA samples in aliquots.

WARNING **CHEMICAL HAZARD. 10 × RT Buffer** may cause eye, skin, and respiratory tract irritation. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Example Experiment

For the example experiment, RNA is extracted from the liver, bladder, and kidney tissues of an individual. RNA concentration is determined spectrophotometrically (using A_{260}), and the RNA is diluted to a final concentration of 50 ng/µL.

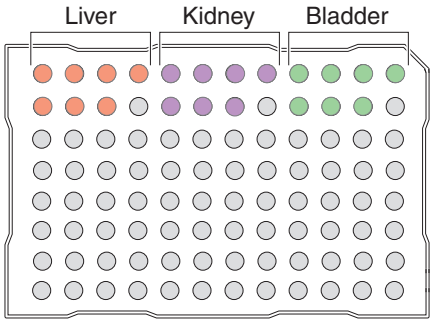
The RT master mix is prepared as follows, using guidelines from the *High Capacity cDNA Archive Kit Protocol*:

Component	µL/Reaction	µL/21 Reactions[a]
10X Reverse Transcription Buffer	10	210
25X dNTPs	4	84
10X random primers	10	210
MultiScribe™ Reverse Transcriptase, 50 U/µL	5	105
Nuclease-free water	21	441
Total per reaction	50	1050

a. Each RT reaction is 100 µL (see below). If you need 5 µL cDNA for each of 104 PCR reactions per tissue (see "Creating a Relative Quantification (RQ) Plate Document" on page 26), you need 6 RT reactions per tissue. Extra volume (enough for one additional RT reaction per tissue) is included to account for pipetting losses, as well as extra cDNA for archiving.

The cDNA archive plate is then prepared by pipetting into each well:

- 50 µL of the RT master mix
- 30 µL of nuclease-free water
- 20 µL of RNA sample (bringing the total starting amount of RNA to 1 µg per 100 µL reaction)



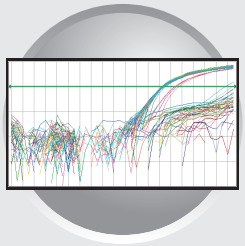
The RNA is then converted to cDNA using the thermal cycling parameters for two-step RT-PCR, as described in "Thermal Profile Parameters for RT" on page 21.

The cDNA is stored at –20 °C until use.

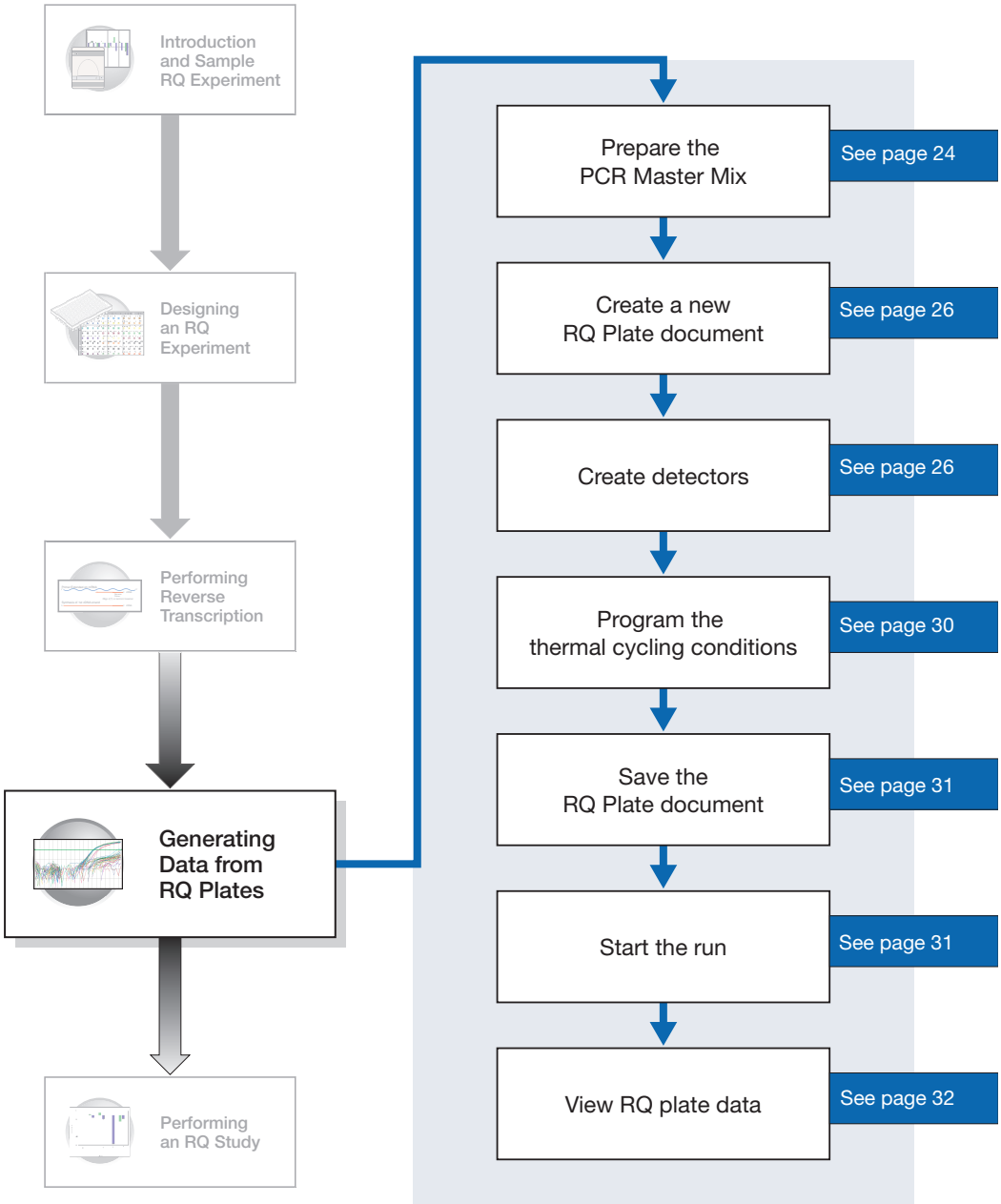
Notes

Chapter 4

Generating Data from RQ Plates



Workflow



Notes

Before You Begin

Check that background and pure-dye runs have been performed regularly to ensure optimal performance of the 7300/7500 system. For more information about calibrating the 7300/7500 system, refer to the Online Help.

Preparing the PCR Master Mix

The second step (PCR) in the two-step RT-PCR procedure is amplifying the cDNA, which you perform using the TaqMan® Universal PCR Master Mix reagents.

The *TaqMan Universal PCR Master Mix Protocol* (PN 4304449) explains how to use the reagents in the kit. The following table lists the universal assay conditions (volume and final concentration) for using the master mix.

CAUTION **CHEMICAL HAZARD. TaqMan Universal PCR Master Mix** may cause eye and skin irritation. Exposure may cause discomfort if swallowed or inhaled. Read the MSDS, and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Reaction Component	µL/ Sample	Final Concentration
TaqMan Universal PCR Master Mix (2×)	25.0	1×
Forward primer	5.0	50 to 900 nM
Reverse primer	5.0	50 to 900 nM
TaqMan probe	5.0	50 to 250 nM
cDNA sample	5.0	10 to 100 ng
Nuclease-free water	5.0	—
Total	50.0	—

If you design probes and primers using Primer Express software, they must be optimized to work with the universal assay conditions, using the volumes listed in the table above. All Assays-by-Design and Assays-on-Demand products are formulated so that the final concentration of the primers and probes are within the recommended values.

Notes

Preparing the Reaction Plate

1. Label the reaction plates, ensuring that you include an endogenous control for each sample type (for example, each tissue in a study comparing multiple tissues). If samples are spread across multiple plates, each plate must have an endogenous control. Additionally, every plate must include an endogenous control for every sample type on the plate.

2. Into each well of the reaction plate, add 50 µL of the appropriate PCR master mix.

3. Keep the reaction plates on ice until you are ready to load them into the 7300/7500 system.

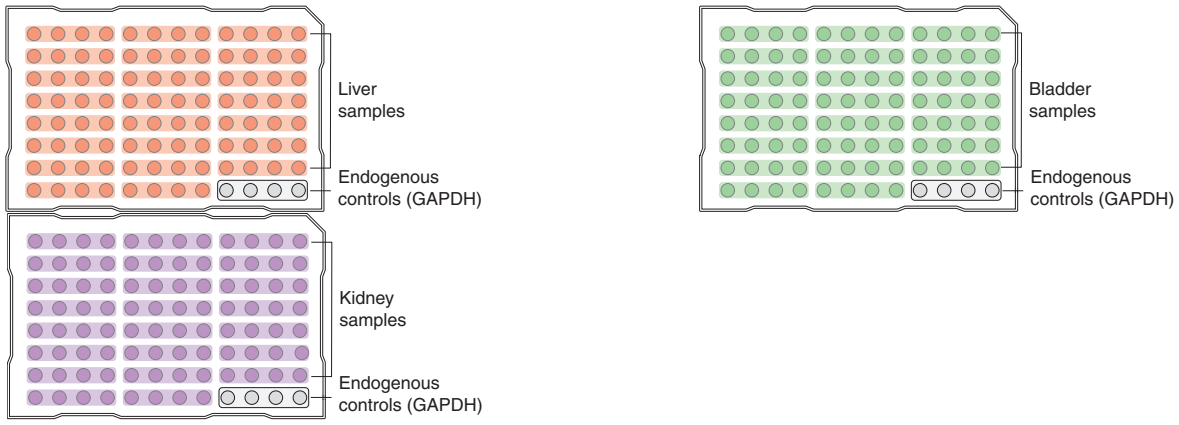
Example Experiment

Primers and probes for the example RQ experiment are obtained from the Assays-on-Demand product line and are provided as a 20× Gene Expression Assay Mix. The PCR master mix is prepared as follows:

Reaction Component	µL/ Sample	µL/ 5 Reactions[b]	Final Concentration
TaqMan Universal PCR Master Mix (2×)	25.0	125.0	1×
20× Assays-on-Demand™ Gene Expression Assay Mix[a]	2.5	12.5	1×
cDNA sample	5.0	25.0	50 ng (for the 50-µL reaction)
Nuclease-free water	17.5	87.5	—
Total	50.0	250	—

a. Contains forward and reverse primers and labeled probe.
b. 24 master mixes are prepared, one for each of 23 genes plus the endogenous control. Volume for five reactions (4 replicates plus extra) to account for pipetting losses.

Samples and endogenous controls are arranged on three plates as shown below. 50 µL of PCR master mix containing cDNA are added to each well.



The reactions are kept on ice until the plates are loaded on the 7300/7500 system.

Notes

Creating a Relative Quantification (RQ) Plate Document

Overview

An RQ Plate document stores data collected from an RQ run for a single plate. There must be one RQ Plate document for every RQ plate. RQ Plate documents also store other information, including sample names and detectors.

Run Setup Requirements

For each RQ plate document that you create, specify detectors, endogenous controls, and detector tasks:

- A detector is a virtual representation of a gene-specific nucleic acid probe reagent used in assays. You specify which detector to use for each target sequence. Appendix A explains how to create detectors.

  IMPORTANT! To conduct a comparative analysis of the data in a study, all the plates in the study must contain a common set of detectors.

- An endogenous control(s) (as defined in "Specifying the Components of an RQ Experiment" on page 13). If your experiment consists of multiple plates, each plate must have at least one endogenous control with at least three replicates. If your experiment consists of a single plate with multiple samples, there must be an endogenous control for each sample. All plates in an RQ experiment must use the same endogenous control (for example, GAPDH).
- A detector task specifies how the software uses the data collected from the well during analysis. You apply one of two tasks to each detector in each well of a plate document.

Task	Symbol	Apply to detectors of...
Target	T	Wells that contain PCR reagents for the amplification of target sequences.
Endogenous Control	E	Wells that contain reagents for the amplification of the endogenous control sequence.

Notes

Creating an RQ Plate Document

You can enter sample information into a new plate document, import sample information from existing plate documents, or use a template document to set up new plate documents. This section describes setting up new plate documents. Refer to the Online Help for information about importing sample information or using template documents.

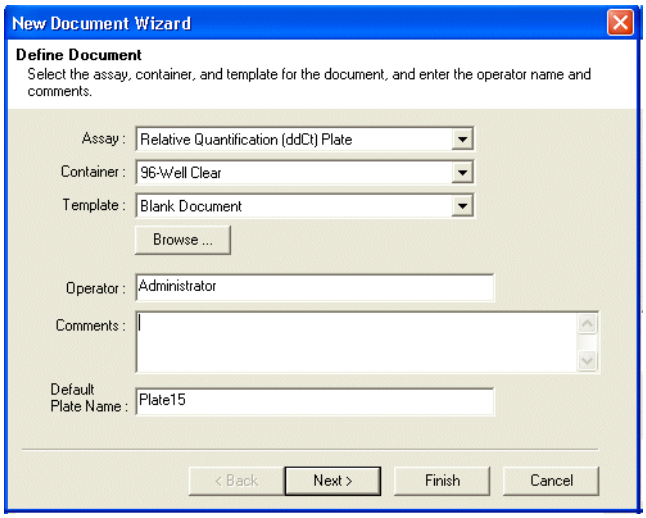
To create a new plate document:

1. Select **Start > Programs > Applied Biosystems 7300/7500 > Applied Biosystems 7300/7500 SDS Software** ( ) to start the SDS software.

2. Select **File** > **New**.

3. In the Assay drop-down list of the New Document Wizard, select **Relative Quantification (ddCt) Plate**. Accept the default settings for Container and Template (**96-Well Clear** and **Blank Document**).

   IMPORTANT! You cannot use RQ Plate documents for AQ assays and vice versa. The information stored in AQ and RQ Plate documents is not interchangeable.



4. Enter a name in the Default Plate Name field, or accept the default.

5. Click **Next >**.

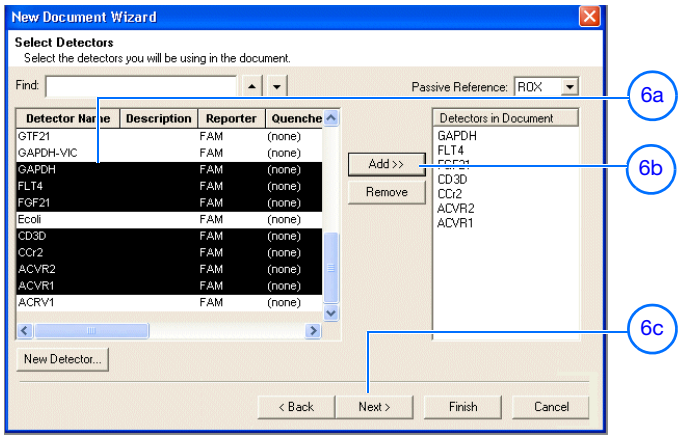
6. Select detectors to add to the plate document.

   a. Click to select a detector. (Ctrl-click to select multiple detectors.) If no detectors are listed in the Detector Manager, create detectors as explained in Appendix A, "Creating Detectors."

   b. Click **Add>>**. The detectors are added to the plate document.

      Note: To remove a detector from the Detectors in Document panel, select the detector, then click **Remove**.

   c. Click **Next >**.



Notes

7. Specify the detectors and tasks for each well.

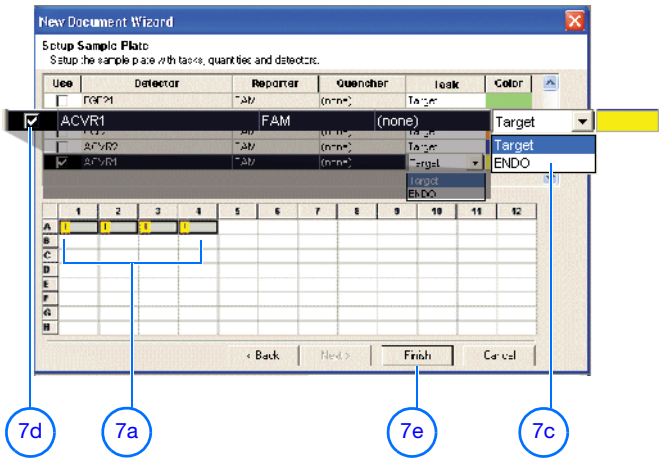
a. Click a well (or group of wells, for replicates) to select it.

b. Click to select the detector(s) for the well.

c. Click under the Task column to assign the detector task.

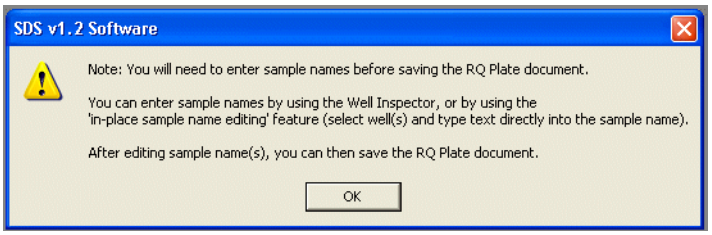
d. Select **Use**.

e. Click **Finish**.



You cannot add RQ plates to RQ studies unless you have specified sample names, as indicated in the message shown to the right. Click **OK**.

The SDS software creates the plate document and displays the Well Inspector.


SDS v1.2 Software
Note: You will need to enter sample names before saving the RQ Plate document.
You can enter sample names by using the Well Inspector, or by using the 'in-place sample name editing' feature (select well(s) and type text directly into the sample name).
After editing sample name(s), you can then save the RQ Plate document.
OK

8. Enter the sample names.

a. In the Well Inspector, click a well or click-drag to select replicate wells.

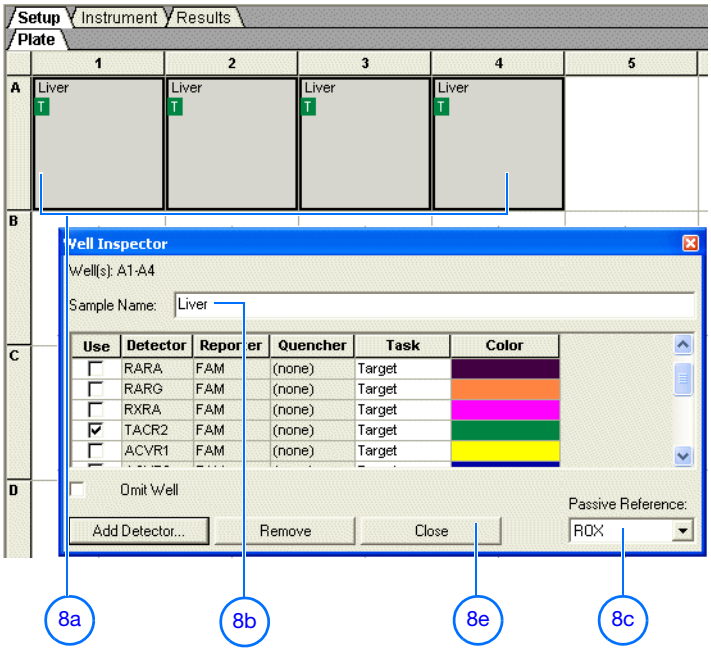
b. Enter the sample name.

c. If necessary, change the setting for the Passive Reference dye. (By default, the ROX™ dye is selected.)

d. Repeat steps a through c until you specify sample names and passive reference dyes for all the wells on the plate.

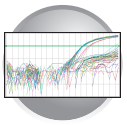
IMPORTANT! If your experiment does not use all the wells on a plate, do not omit the wells from use at this point. You can omit unused wells after the run. For information about omitting unused wells, refer to the Online Help.

Note: You can change the sample setup information (sample name, detector, task) after a run is complete.



e. Close the Well Inspector.

Notes



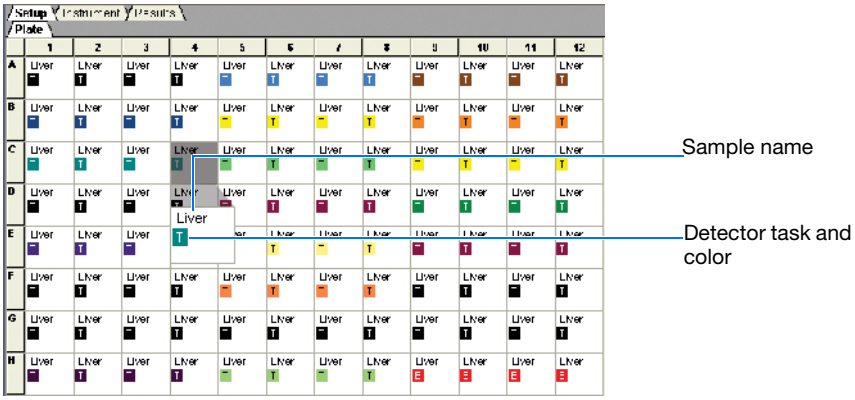
9. Verify the information on each well in the Setup tab.

Example Experiment

In the example RQ experiment, the samples for each of the three tissues (liver, kidney, and bladder) are loaded on three separate plates. Consequently, three RQ Plate documents are created, one for each of the sample plates.

Because the experiment is singleplex, there is only one sample—either a target or endogenous control—in each well. Each well is associated with a detector (indicated by the colored squares). Additionally, each well is assigned a detector task—T (target) or E (endogenous control).

The figure below shows the example RQ Plate document after sample names, detectors, and detector tasks are assigned for each well in the liver plate.



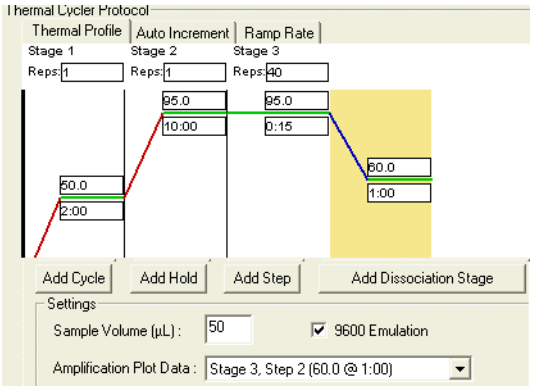
Notes

Specifying Thermal Cycling Conditions and Starting the Run

Default Thermal Cycling Conditions for PCR

If you selected the two-step RT-PCR method for your RQ experiment (recommended), you have already completed the RT step and are ready to PCR amplify cDNA.

The default thermal cycling conditions for the PCR step of the procedure, shown in the following table, should appear in the Instrument tab.



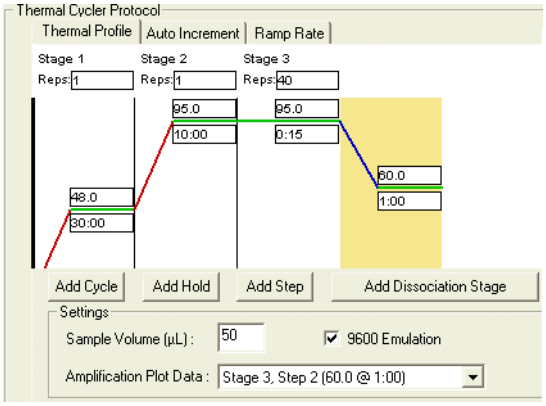
Times and Temperatures (Two-step RT-PCR)				
1) RT Step	HOLD	HOLD	* For reference only. RT is complete at this point.	
	10 min @ 25 °C	120 min @ 37 °C		
2) PCR Step	Initial Steps		PCR (Each of 40 cycles)	
	AmpErase® UNG Activation	AmpliTaq Gold® DNA Polymerase Activation	Melt	Anneal/Extend
	HOLD	HOLD	CYCLE	
	2 min @ 50 °C	10 min @ 95 °C	15 sec @ 95 °C	1 min @ 60 °C

Thermal Cycling Conditions for One-Step RT-PCR

If you select the one-step RT-PCR method, cDNA generation and amplification take place simultaneously at this point in the workflow.

The following table shows the thermal cycling conditions for one-step RT-PCR experiments.

Note: Refer to the Online Help for instructions on modifying thermal cycling parameters.



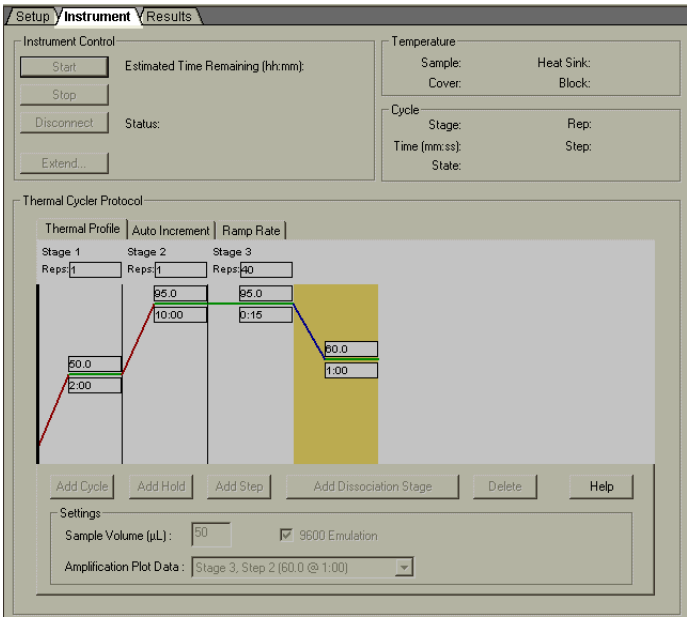
Times and Temperatures (One-step RT-PCR)			
Initial Steps		PCR (Each of 40 Cycles)	
Reverse Transcription	AmpliTaq® Gold DNA Polymerase Activation	Melt	Anneal/Extend
HOLD	HOLD	CYCLE	
30 min @ 48 °C	10 min @ 95 °C	15 sec @ 95 °C	1 min @ 60 °C

Notes

To specify thermal cycling conditions and start the run:

1. Select the **Instrument** tab.

   By default, the standard PCR conditions for the PCR step of the two-step RT-PCR method are displayed.



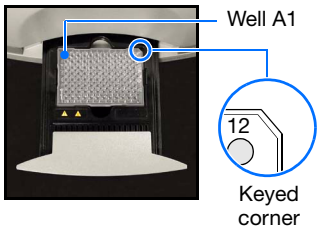
2. Verify that:
   - For two-step RT-PCR, the default PCR thermal cycling conditions are set.
   - For one-step RT-PCR, you set the thermal cycling parameters as shown in "Thermal Cycling Conditions for One-Step RT-PCR" on page 30.
   - Sample volume is 50 µL.
   - 9600 Emulation is selected.

   Note: If you are using SYBR Green I chemistry and you want to determine if there is contamination or if you want to determine the dissociation temperature, create a separate Dissociation assay or template. Refer to the Online Help for more information.

   Note: In the 7300 instrument, the 9600 Emulation feature is not available.

3. Select **File** > **Save As**, enter a name for the RQ Plate document, then click **Save**.

4. Load the plate into the instrument..



   Note: The A1 position is in the top-left side of the instrument tray.

5. Click **Start**.

   As the instrument performs the PCR run, it displays real-time status information in the Instrument tab and records the fluorescence emissions.

   After the run, a message indicates whether or not the run is successful.

   All data generated during the run are saved to the RQ Plate document that you specified in step 3.

Notes

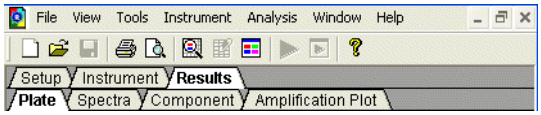
Analyzing and Viewing RQ Plate Data

Starting the Analysis

To analyze RQ Plate data after the run, click ▶ or select **Analysis > Analyze**. The SDS software mathematically transforms the raw fluorescence data to establish a comparative relationship between the spectral changes in the passive reference dye and those of the reporter dyes. Based on that comparison, the software generates four result views: Plate, Spectra, Component, and Amplification Plot.

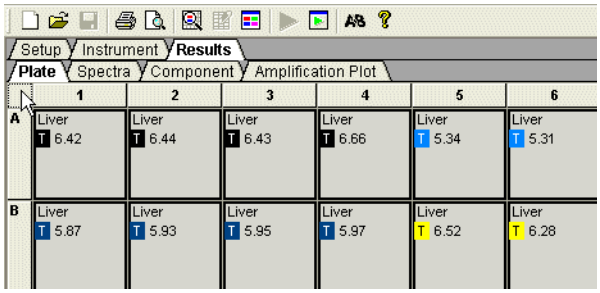
About the Results Tab

In the Results tab, you can view the results of the run and change the parameters. For example, you can omit samples or manually set the baseline and threshold. If you change any parameters, you should reanalyze the data.

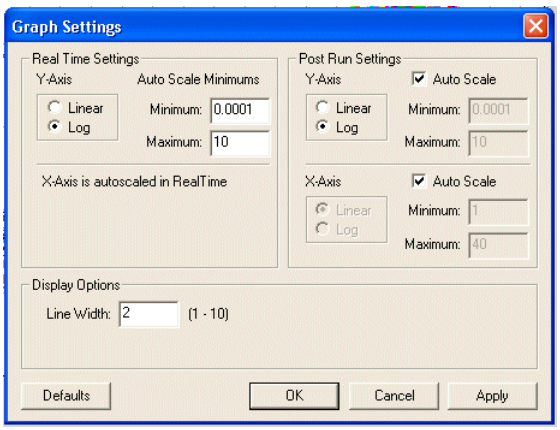

File View Tools Instrument Analysis Window Help
Setup Instrument Results
Plate Spectra Component Amplification Plot

The Results tab has four secondary tabs, each of which is described below. Details are provided in the Online Help.

- To move between views, click a tab.
- To select all 96 wells on a plate, click the upper-left corner of the plate.


Setup Instrument Results
Plate Spectra Component Amplification Plot
1 2 3 4 5 6
A Liver T 6.42 Liver T 6.44 Liver T 6.43 Liver T 6.66 Liver T 5.34 Liver T 5.31
B Liver T 5.87 Liver T 5.93 Liver T 5.95 Liver T 5.97 Liver T 6.52 Liver T 6.28

- To adjust graph settings, click the y- or x-axes of a plot to display the Graph Settings dialog. The adjustable settings depend on which plot you are viewing.

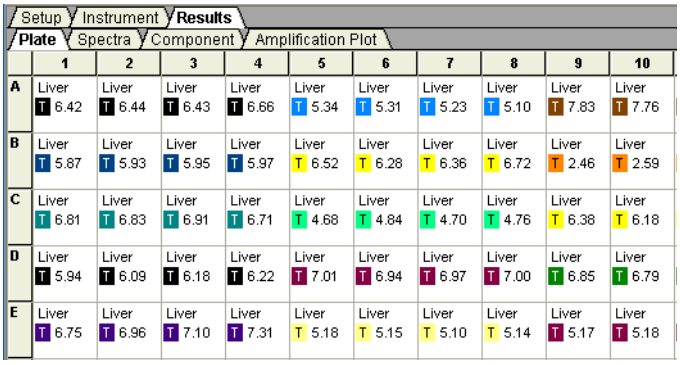


Notes

Plate Tab

Displays the results data of each well, including the:

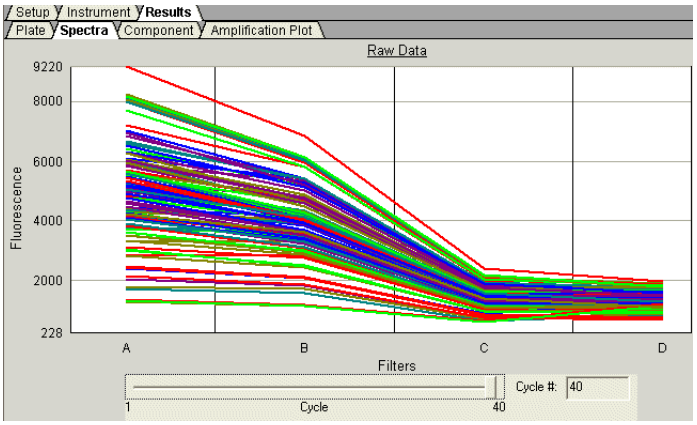
- Sample name and detector task and color for each well
- Calculated R_n value


Setup Instrument Results
Plate Spectra Component Amplification Plot
1 2 3 4 5 6 7 8 9 10
A Liver T 6.42 Liver T 6.44 Liver T 6.43 Liver T 6.66 Liver T 5.34 Liver T 5.31 Liver T 5.23 Liver T 5.10 Liver T 7.83 Liver T 7.76
B Liver T 5.87 Liver T 5.93 Liver T 5.95 Liver T 5.97 Liver T 6.52 Liver T 6.28 Liver T 6.36 Liver T 6.72 Liver T 2.46 Liver T 2.59
C Liver T 6.81 Liver T 6.83 Liver T 6.91 Liver T 6.71 Liver T 4.68 Liver T 4.84 Liver T 4.70 Liver T 4.76 Liver T 6.38 Liver T 6.18
D Liver T 5.94 Liver T 6.09 Liver T 6.18 Liver T 6.22 Liver T 7.01 Liver T 6.94 Liver T 6.97 Liver T 7.00 Liver T 6.85 Liver T 6.79
E Liver T 6.75 Liver T 6.96 Liver T 7.10 Liver T 7.31 Liver T 5.18 Liver T 5.15 Liver T 5.10 Liver T 5.14 Liver T 5.17 Liver T 5.18

Spectra Tab

Displays the fluorescence spectra of selected wells.

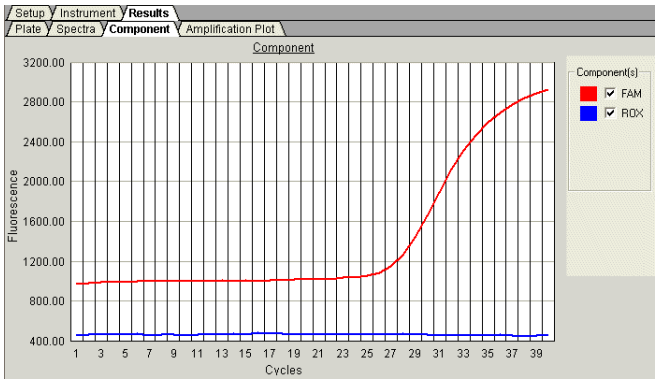
- The Cycles slider allows you to see the spectra for each cycle by dragging it with the pointer.
- The Cycle # text box shows the current position of the slider.



Component Tab

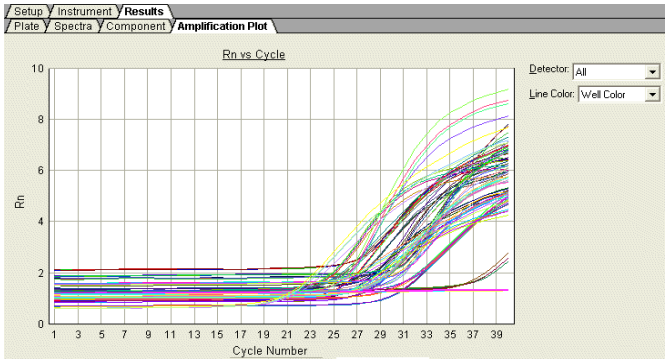
Displays the complete spectral contribution of each dye in a selected well over the duration of the PCR run. Only the first selected well is shown at one time.

Note: If you are using TaqMan® products, three components (ROX® dye, reporter dye, and TAMRA™ quencher) are displayed in the Component tab. If you are using TaqMan® MGB products, only two components (ROX and reporter dyes) are displayed, as shown in the figure on the right.



Amplification Plot Tab

Displays a plot of R_n as a function of cycle number for the selected detector and well(s).



Notes

Reanalyzing Data

Raw fluorescence data (spectra), R_n values, and well information (sample name, detector, and detector task) are saved in an RQ plate document.

If you decide to omit wells or change well information after a run is complete, you must reanalyze the data.

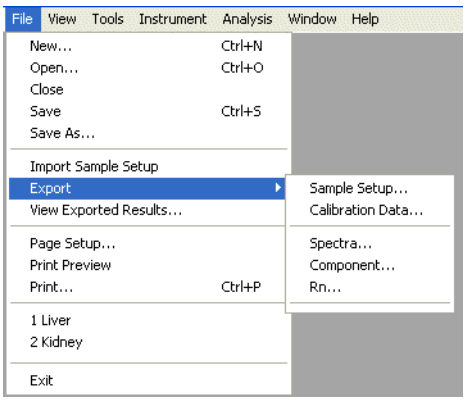
Note: After the software analyzes data, the Analyze button is disabled ( ). Whenever you change a setting that requires reanalysis, the Analyze button is enabled ( ).

Exporting RQ Plate Data

You can export numeric data from RQ plates into text files, which can then be imported into spreadsheet applications such as Microsoft Excel.

1. Select **File** > **Export**, then select the data type to export:
   - **Sample Setup** (*.txt)
   - **Calibration Data** (*.csv)
   - **Background Spectra** (*.csv)
   - **Component** (*.csv)
   - **Rn** (*.csv)

   Typically, you export sample setup data for newly created and newly run plates; other data types are exported for existing plates.



2. Enter a file name for the export file.

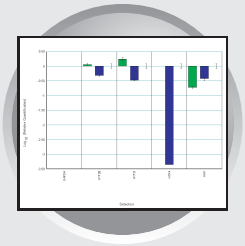
   Note: The name of the dialog box depends on the type of data you want to export.

3. Click **Save**.

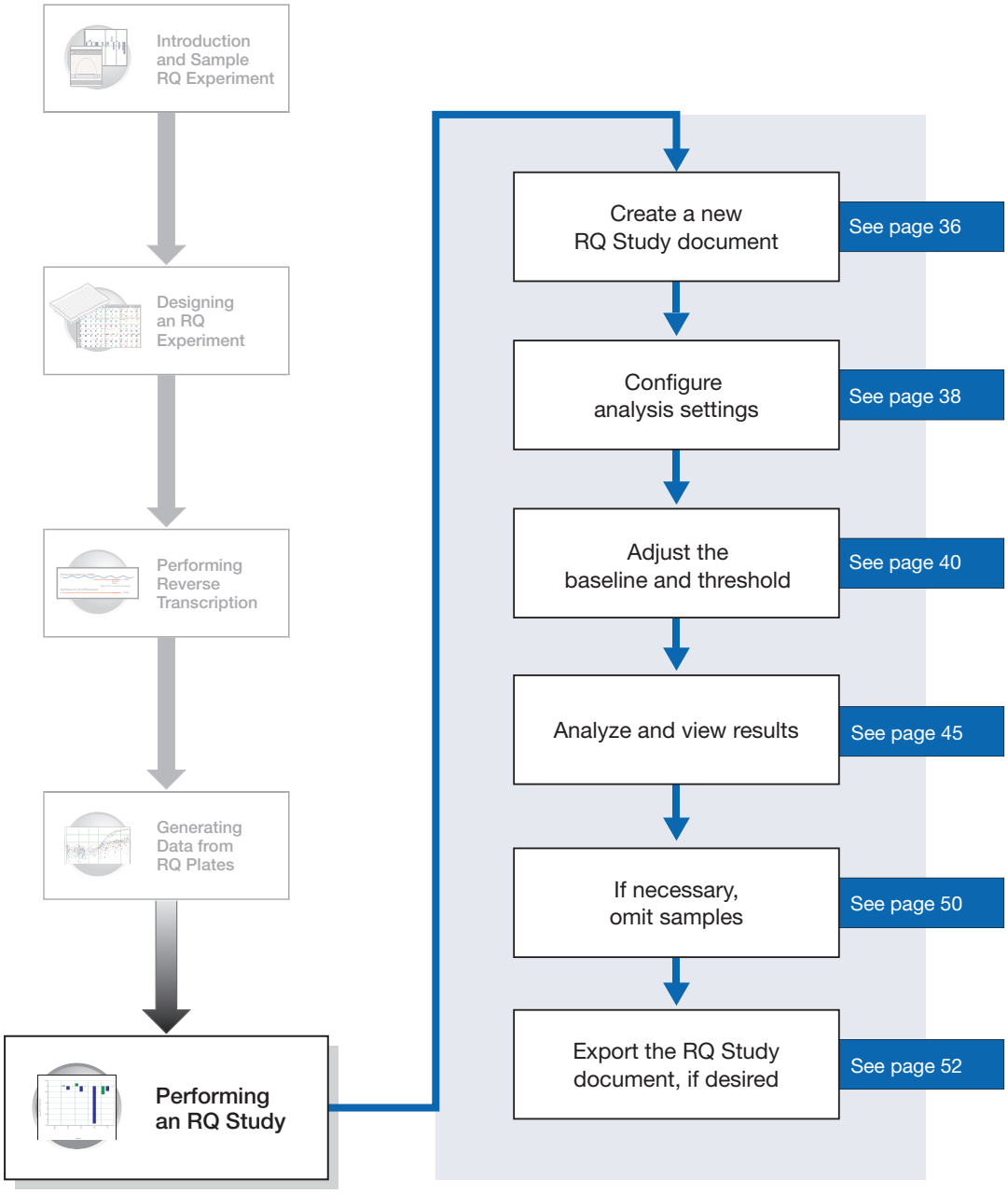
Notes

Chapter 5

Analyzing Data in an RQ Study



Workflow


Introduction and Sample RQ Experiment
Designing an RQ Experiment
Performing Reverse Transcription
Generating Data from RQ Plates
Performing an RQ Study
Create a new RQ Study document
See page 36
Configure analysis settings
See page 38
Adjust the baseline and threshold
See page 40
Analyze and view results
See page 45
If necessary, omit samples
See page 50
Export the RQ Study document, if desired
See page 52

Notes

Creating an RQ Study Document

To conduct a comparative analysis of RQ plates in a study, you must first create an RQ Study document.

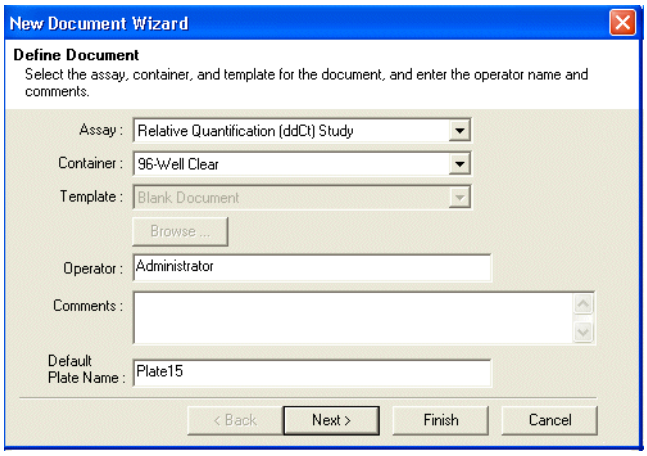
IMPORTANT! RQ Study software is an optional package for the 7300 instrument but is standard for the 7500 instrument.

The SDS software uses the comparative method ($2^{-\Delta\Delta Ct}$) of relative quantification. For more information about methods of calculating relative quantification, refer to *ABI PRISM® 7700 Sequence Detection System User Bulletin #2 (*PN 4303859).

In an RQ study, you can...	You cannot
• Select the endogenous control and the calibrator sample. • Select the control type when applicable. • Set baseline and threshold values and RQ Min/Max Confidence Levels. • Omit individual wells or sample replicates.	• Create, add, or modify samples. • Create, add, or modify detectors. • Change detector tasks. (You can perform these operations in RQ Plate documents.)

To create a new RQ Study document:

1. Select **File**> **New**.
2. In the Assay drop-down list of the New Document Wizard, select **Relative Quantification (ddCt) Study**. Accept the default settings for Container and Template (**96-Well Clear** and **Blank Document**).
3. Enter a name in the Default Plate Name field, or accept the default.
4. Click **Next>**.



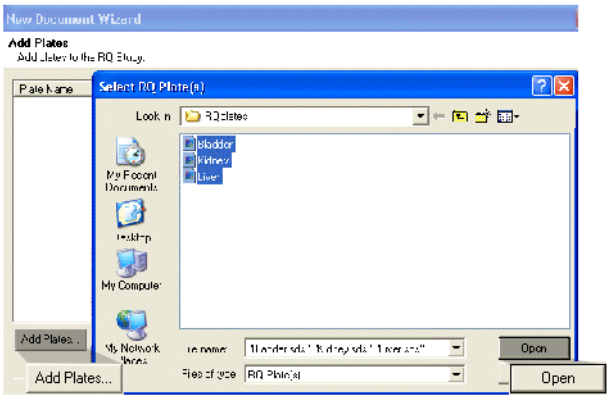
Notes

5. Add RQ plates to the study.

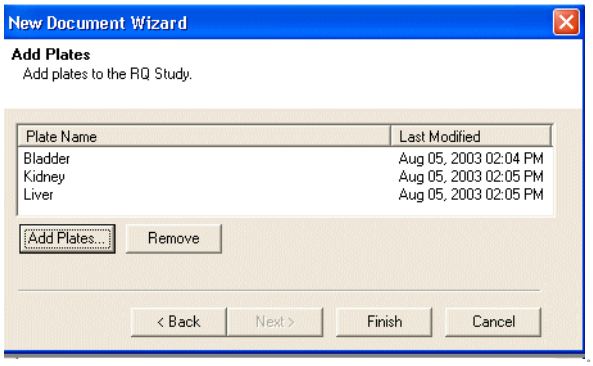
   a. Click **Add Plates**.

   Note: You can add up to 10 RQ plates to an RQ study.

   b. Select the plate(s) that you want to add to the study, then click **Open**.



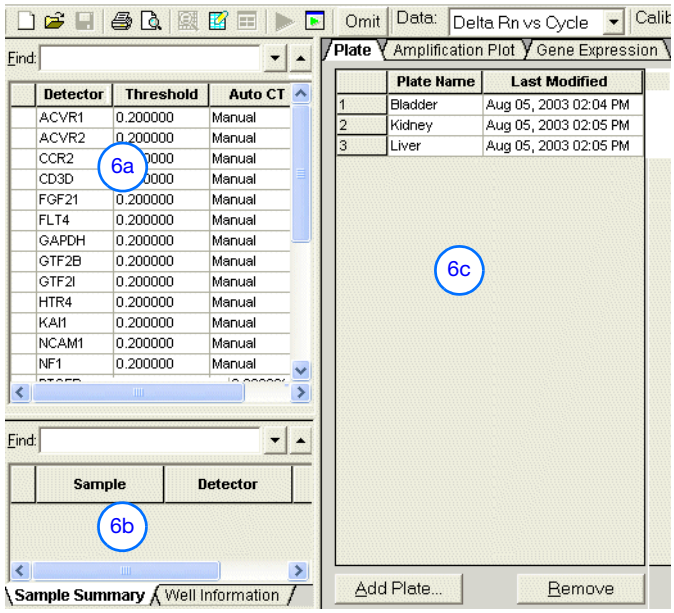
   The selected plates are displayed.



   IMPORTANT! All plates added to a study must have identical thermal cycling parameters—the same number of steps, cycles, sample volume, emulation mode. The SDS software will reject a plate if it detects any differences. (The first plate added to the study serves as the reference plate against which other plates are compared.)

6. Click **Finish**. If desired, save the RQ Study document when prompted.

   The SDS software opens a new RQ Study document and displays the RQ Study main view with its three panes:



   a. RQ Detector grid – Allows you to select detectors to associate with the loaded study. For each detector,Color, Detector name, Threshold value, Auto Ct, and Baseline are displayed.

   Note: At this point, all the values in the Threshold, Auto Ct, and Baseline columns are set to the default values (0.200000, Manual, and [6,15], respectively).

   b. RQ Sample grid – Displays the samples associated with the selected detector(s). The Sample Grid displays numerical results of RQ calculations and has two subtabs: Sample Summary and Well Information.

Notes

c. RQ Results panel – Contains the three results-based tabs: Plate (default), Amplification Plot, and Gene Expression.

Note: You can save the RQ Study document now, or wait until after specifying analysis settings and analyzing the data.

Configuring Analysis Settings

After you create the RQ Study document, you must specify parameter values for the analysis.

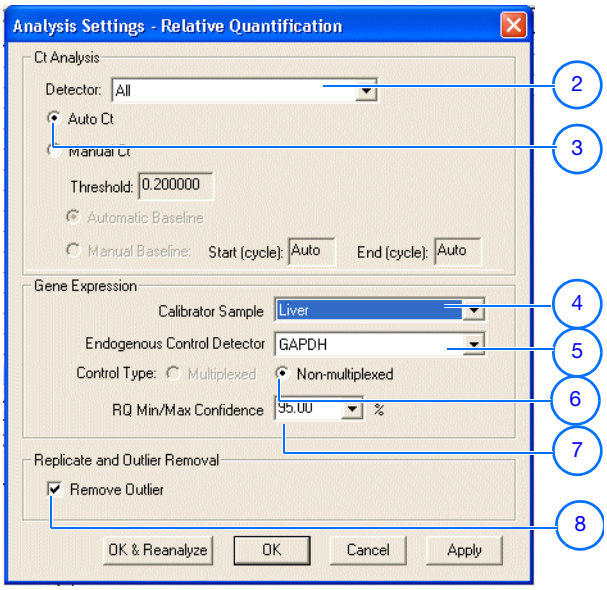
Unless you have already determined the optimal baseline and threshold settings for your experiment, use the automatic baseline and threshold feature of the SDS software (auto Ct), explained below. If the baseline and threshold were called correctly for each well, you can proceed to view the results. Otherwise, you must manually set the baseline and threshold as explained in "Manual Baseline and Threshold Determination" on page 40.

To configure analysis settings:

1. Click or select **Analysis** > **Analysis Settings.**
2. In the Detector drop-down list, select **All**.
3. Select **Auto Ct**. The SDS software automatically generates baseline and threshold values for each well.

IMPORTANT! After analysis, you must verify that the baseline and threshold were called correctly for each well, as explained in "Adjusting the Baseline and Threshold" on page 40.

Alternatively, you can select Manual Ct and specify the threshold and baseline manually.



Notes

4. Select the Calibrator Sample.

   Note: If your experiment uses only a single plate, there must be at least two different samples that have different names and have their own endogenous controls. (You can go back to a saved RQ Plate document and change the sample names, if necessary.)

5. Select the Endogenous Control Detector.

6. Select the Control Type if the study contains both multiplex and nonmultiplex reactions.

   Note: The Multiplexed or Non-Multiplexed options are active only if the plates loaded for analysis contain both multiplexed and nonmultiplexed reactions that share the same endogenous control.

7. Select the RQ Min/Max Confidence level.

   Note: The SDS software uses this value to calculate error bars for gene expression levels, as explained in "Error Bars for Gene Expression Plots" on page 48.

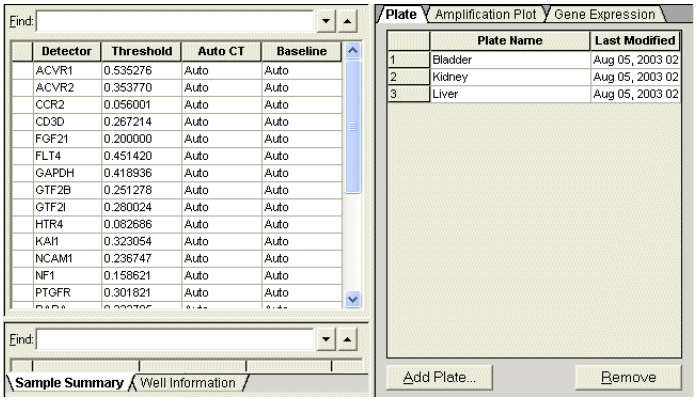
8. Optionally, select **Remove Outliers** to enable the SDS software to automatically identify and filter outliers for groups containing at least four replicates.

   Note: You can also remove outliers manually, as explained in "Omitting Samples from a Study" on page 50.

9. Click **OK & Reanalyze**. The detector information appears in the RQ Detector grid.

   After analysis, the Threshold column displays the automatically calculated threshold values. The Auto Ct and Baseline columns are set to "Auto."

   For more information about the settings in the Analysis Settings dialog box, refer to the Online Help.



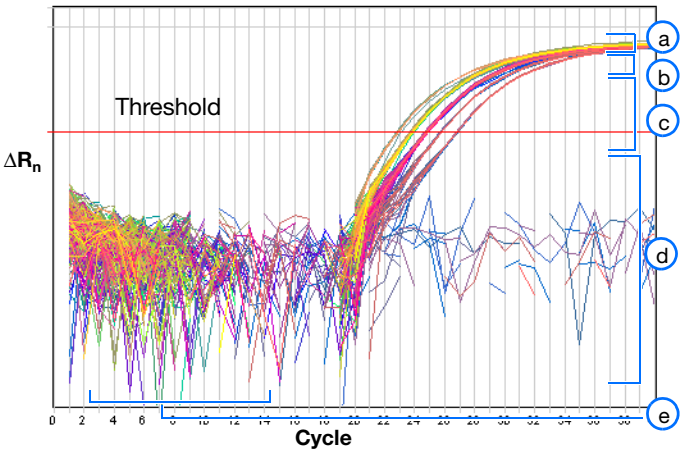
Notes

After the analysis, you must verify that the baseline and threshold were called correctly for each detector, as explained in the following section.

Adjusting the Baseline and Threshold

Automatic Baseline and Threshold Determination

The SDS software calculates baseline and threshold values for a detector based on the assumption that the data exhibit the "typical" amplification curve.



A typical amplification curve has a:

- Plateau phase (a)
- Linear phase (b)
- Geometric phase (c)
- Background (d)
- Baseline (e)

Experimental error (such as contamination, pipetting errors, and so on) can produce data that deviate significantly from data for typical amplification curves. Such atypical data cause the software algorithm to generate incorrect baseline and threshold values for the associated detector.

Therefore, Applied Biosystems recommends reviewing all baseline and threshold values after analysis of the study data. If necessary, adjust the values manually as described on page 43.

Manual Baseline and Threshold Determination

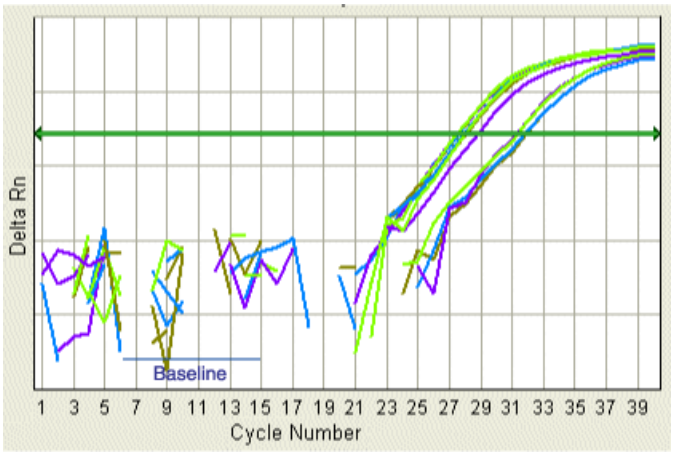
If you set the baseline and threshold values manually for any detector in the study, you must perform the procedure on page 43 for each of the detectors.

The following amplification plots show the effects of baseline and threshold settings.

Notes

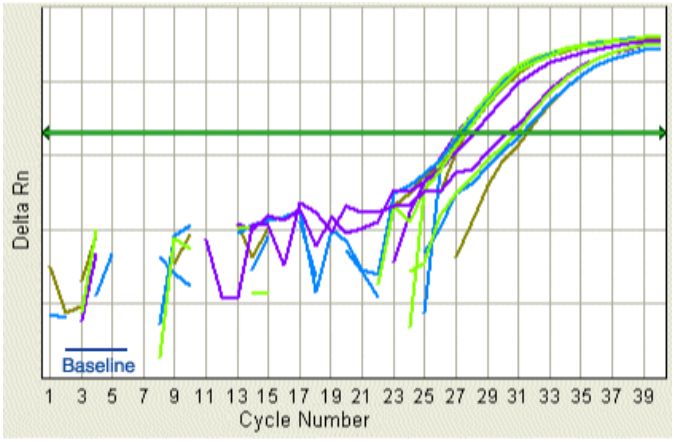
Baseline Set Correctly

The amplification curve begins after the maximum baseline. No adjustment necessary.



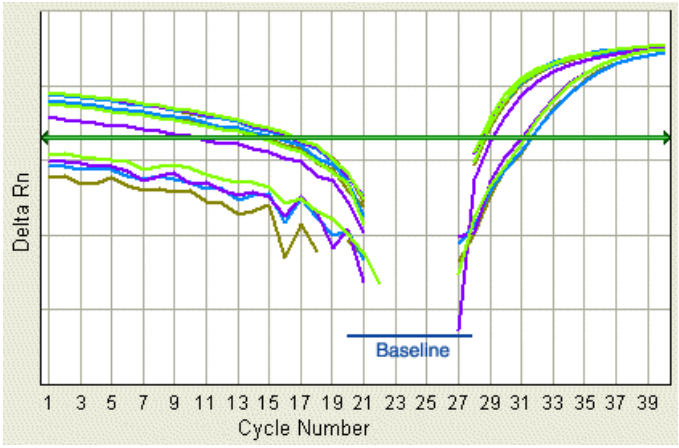
Baseline Set Too Low

The amplification curve begins too far to the right of the maximum baseline. Increase the End Cycle value.



Baseline Set Too High

The amplification curve begins before the maximum baseline. Decrease the End Cycle value.



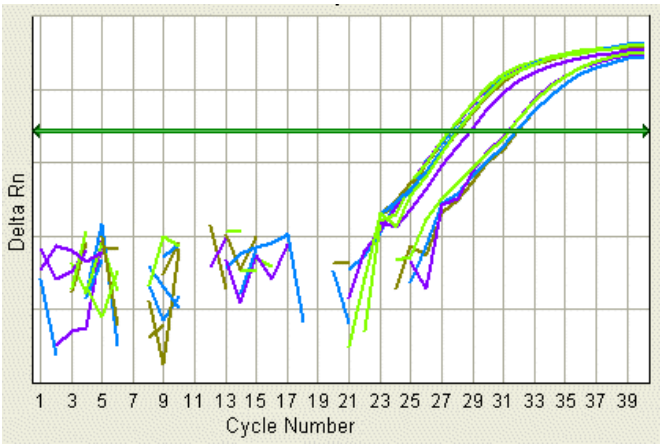
Notes

Threshold Set Correctly

The threshold is set in the geometric phase of the amplification curve.

Threshold settings above or below the optimum increase the standard error of the replicate groups.

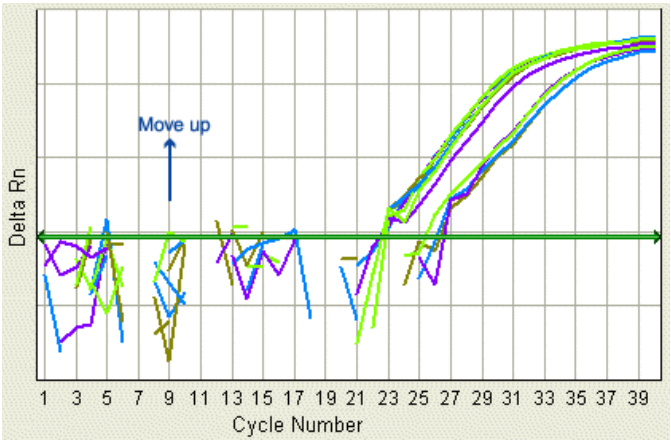
	Sample	Detector	Task	Avg Ct	Avg dCt	dCt std err	ddCt	RQ	P Value	RQ Min	RQ Max	Outl Rem
	Liver	CD3D	Target	27.492	6.369	0.111	0.000	1.000	1.000	0.828	1.208	0
	Kidney	CD3D	Target	28.203	8.377	0.207	2.008	0.249	0.002	0.175	0.353	0
	Bladder	CD3D	Target	31.585	10.216	0.193	3.847	0.070	0.002	0.050	0.096	0



Threshold Set Too Low

The threshold is set below the geometric phase of the amplification curve. The standard error is significantly higher than that for a plot where the threshold is set correctly. Drag the threshold bar up into the geometric phase of the curve.

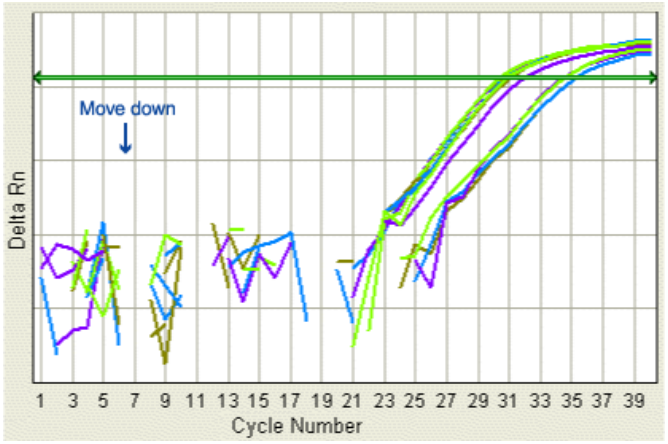
	Sample	Detector	Task	Avg Ct	Avg dCt	dCt std err	ddCt	RQ	P Value	RQ Min	RQ Max	Outl Rem
	Liver	CD3D	Target	21.117	-0.006	0.246	0.000	1.000	1.000	0.659	1.517	0
	Kidney	CD3D	Target	21.514	1.688	0.264	1.694	0.309	0.002	0.198	0.483	0
	Bladder	CD3D	Target	24.043	2.673	0.281	2.679	0.156	0.002	0.097	0.252	0



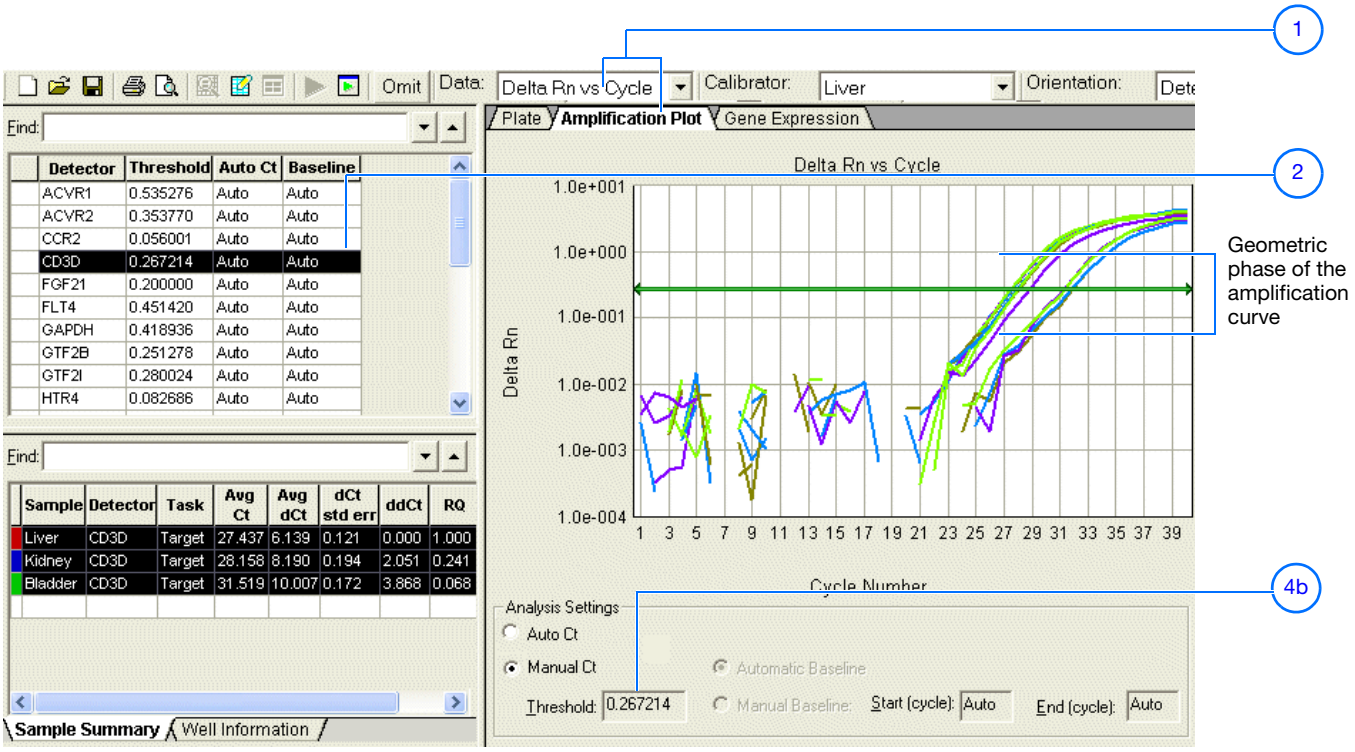
Threshold Set Too High

The threshold is set above the geometric phase of the amplification curve. The standard error is significantly higher than that for a plot where the threshold is set correctly. Drag the threshold bar down into the geometric phase of the curve.

	Sample	Detector	Task	Avg Ct	Avg dCt	dCt std err	ddCt	RQ	P Value	RQ Min	RQ Max	Outl Rem
	Liver	CD3D	Target	33.449	12.327	0.114	0.000	1.000	1.000	0.824	1.214	0
	Kidney	CD3D	Target	34.493	14.667	0.449	2.340	0.197	0.002	0.092	0.423	0
	Bladder	CD3D	Target	38.314	16.944	0.377	4.617	0.041	0.002	0.021	0.077	0



Notes



Detector	Threshold	Auto Ct	Baseline
ACVR1	0.535276	Auto	Auto
ACVR2	0.353770	Auto	Auto
CCR2	0.056001	Auto	Auto
CD3D	0.267214	Auto	Auto
FGF21	0.200000	Auto	Auto
FLT4	0.451420	Auto	Auto
GAPDH	0.418936	Auto	Auto
GTF2B	0.251278	Auto	Auto
GTF2I	0.280024	Auto	Auto
HTR4	0.082686	Auto	Auto

Sample	Detector	Task	Avg Ct	Avg dCt	dCt std err	ddCt	RQ
Liver	CD3D	Target	27.437	6.139	0.121	0.000	1.000
Kidney	CD3D	Target	28.158	8.190	0.194	2.051	0.241
Bladder	CD3D	Target	31.519	10.007	0.172	3.868	0.068

To manually adjust the baseline and threshold:

1. Select the **Amplification Plot** tab, then select **Delta Rn vs. Cycle** in the Data drop-down list.

2. In the RQ Detector grid, select a detector.

   The SDS software displays the:

   - Associated samples (from all plates included in the study) in the RQ Sample grid.
   - Graph for the selected detector in the RQ Results panel.

   Note: When manually adjusting baseline and threshold settings, you can select only one detector at a time. If you select multiple detectors, the Analysis Settings section and the threshold bar are disabled.

3. Set the baseline for the detector.

   a. Under Analysis Settings, select **Manual Baseline**.

Notes

b. Enter values in the Start Cycle and End Cycle fields, ensuring that the amplification curve growth begins at a cycle after the End Cycle value.

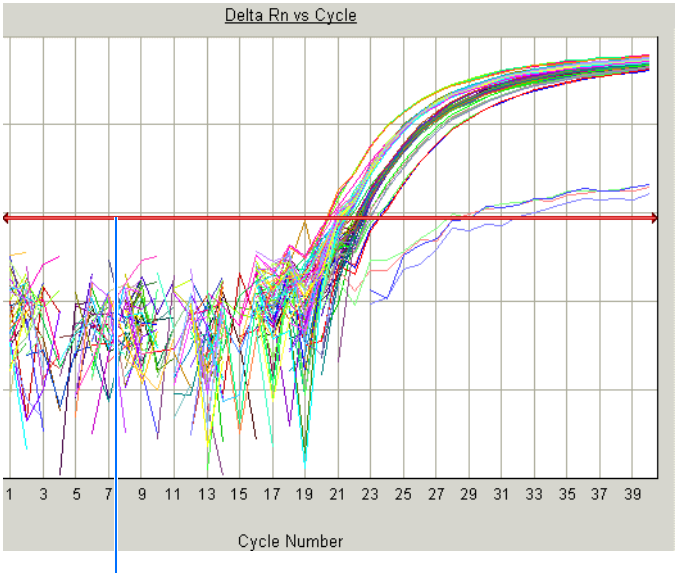
Note: After you change a baseline or threshold setting for a detector, the Analyze button ( ) is enabled, indicating that you must reanalyze the data.

4. Set the threshold for the detector.
   a. Under Analysis Settings, select **Manual Ct**.
   b. Drag the threshold setting bar so the threshold is:
      - Above the background
      - Below the plateaued and linear regions of the amplification curve
      - Within the geometric phase of the amplification curve

      The SDS software adjusts the theshold value and displays it in the Threshold field after reanalyzing.

5. Repeat steps 2 through 3 to set the baseline and threshold values for all remaining detectors in the study.

6. Click or select **Analysis > Analyze** to reanalyze the data using the adjusted baseline and threshold values.

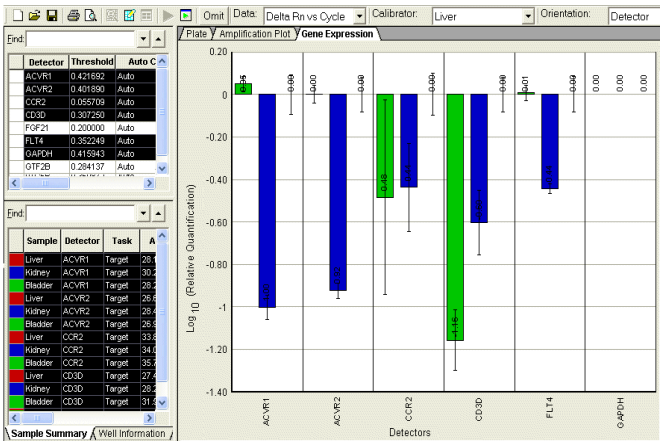


Click and drag the Threshold setting to adjust the threshold. The bar turns red, indicating that the threshold has been changed.

Notes

Analyzing and Viewing the Results of the RQ Study

Selecting Detectors to Include in Results Graphs



In the RQ Detector Grid, select detectors to include in the result graphs by clicking a detector. (Ctrl-click to include multiple detectors; Click-drag to include multiple adjacent detectors.)

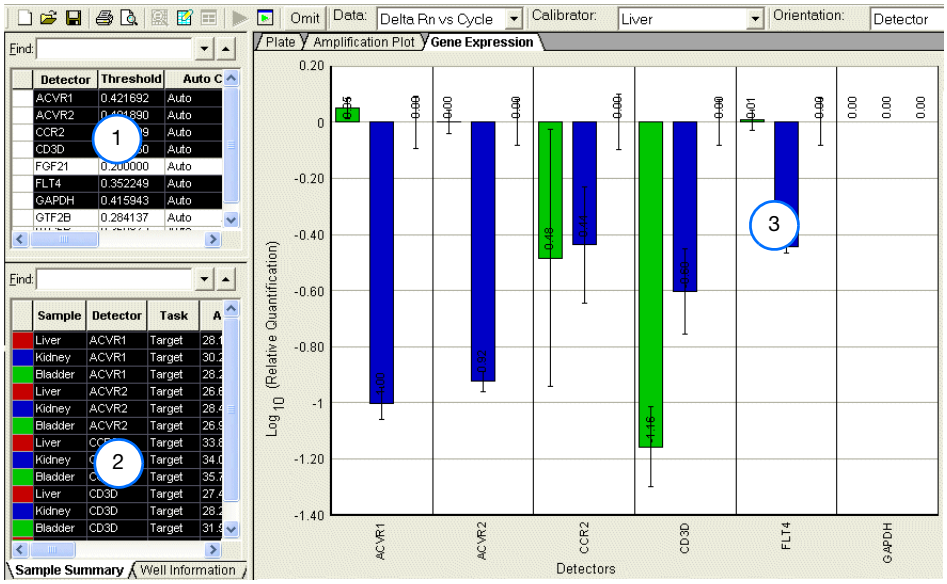
The corresponding samples appear in the RQ Sample Grid. Depending on which tab you select in the RQ Results Panel (Plate, Amplification Plot, or Gene Expression), analysis results are displayed.

To see information about a specific well, select the **Well Information** tab.

Example Experiment

Suppose that you want to view the comparative gene expression levels of the following genes when the liver tissue is used as the calibrator: ACVR1, ACVR2, CCR2, CD3D, and FLT4. Selecting the detectors in the RQ Detector grid (1) displays the sample information in the RQ Sample grid (2) and in a result graph in the RQ Results panel (3). Note that:

- The Gene Expression tab is selected, and the gene expression levels are sorted by detector.
- Gene expression levels for bladder samples are indicated by the green bar; those for kidney samples by the blue bar. These colors also indicate the samples in the RQ Sample Grid and the RQ Results Panel plots.
- Because liver samples are used as calibrators, the expression levels are set to 1. But because the gene expression levels were plotted as $\log_{10}$ values (and the $\log_{10}$ of 1 is 0), the expression level of the calibrator samples appear as 0 in the graph.
- Because the relative quantities of the targets are normalized against the relative quantities of the endogenous control, the expression level of the endogenous control is 0; there are no bars for GAPDH.
- Fold-expression changes are calculated using the equation $2^{-\Delta\Delta CT}$.



Notes

5

Amplification Plot

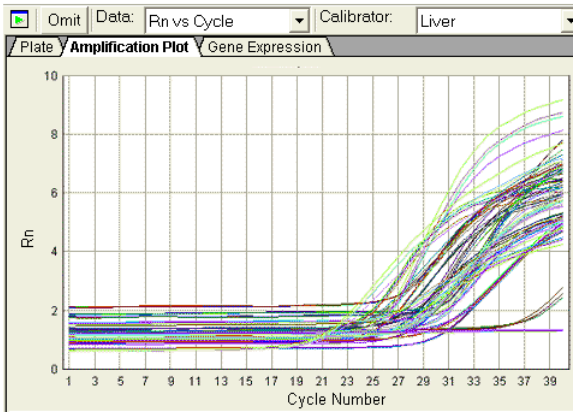
The three Amplification Plots allow you to view post-run amplification of specific samples. The Amplification Plots display all samples for selected detectors.

You can adjust graph settings by clicking the y- or x-axes of a plot to display the Graph Settings dialog, as shown on page 32.

Rn vs. Cycle (Linear) View

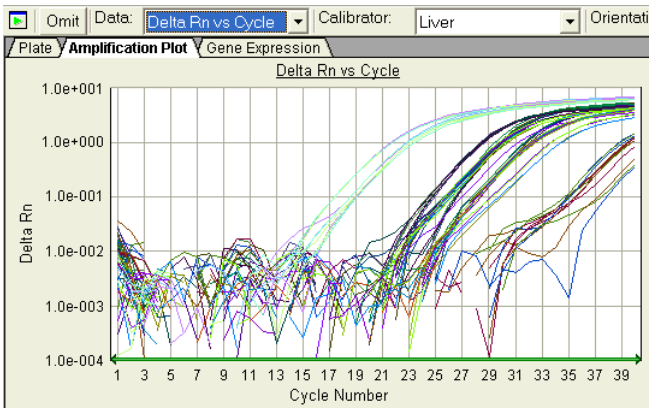
Displays normalized reporter dye fluorescence (R_n) as a function of cycle. You can use this plot to identify and examine irregular amplification.

For more information about R_n, refer to the *SDS Chemistry Guide*.



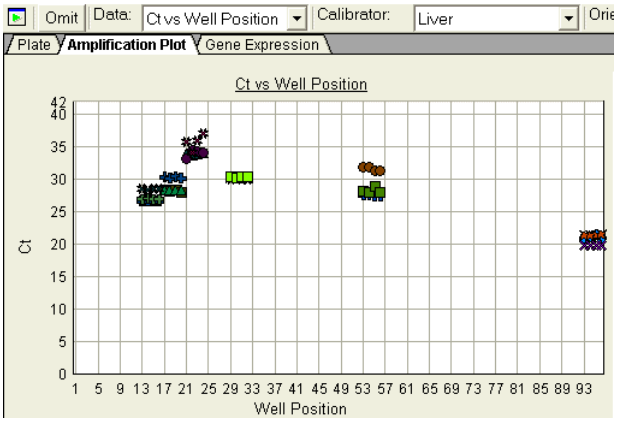
ΔRn vs. Cycle (Log) View

Displays dye fluorescence (ΔR_n) as a function of cycle number. You can use this plot to identify and examine irregular amplification and to manually set the threshold and baseline parameters for the run.



Ct vs. Well Position View

Displays threshold cycle (C_T) as a function of well position. You can use this plot to locate outliers from detector data sets (see "Omitting Samples from a Study" on page 50 for more information).



Notes

Gene Expression Plot

Gene Expression plots show the expression level or fold-difference of the target sample relative to the calibrator.

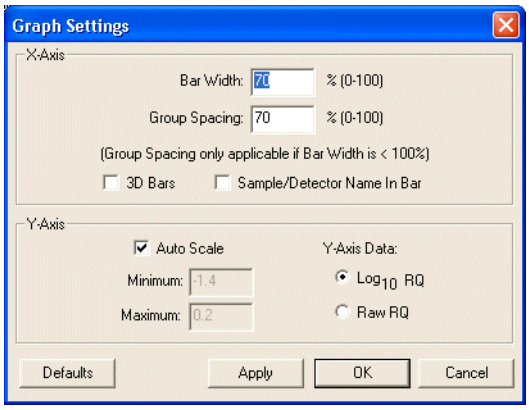
Because the calibrator is compared to itself, the expression level for the calibrator is always 1.

Adjusting Graph Settings

You can adjust graph settings for gene expression plots in the Graph Settings dialog box, including:

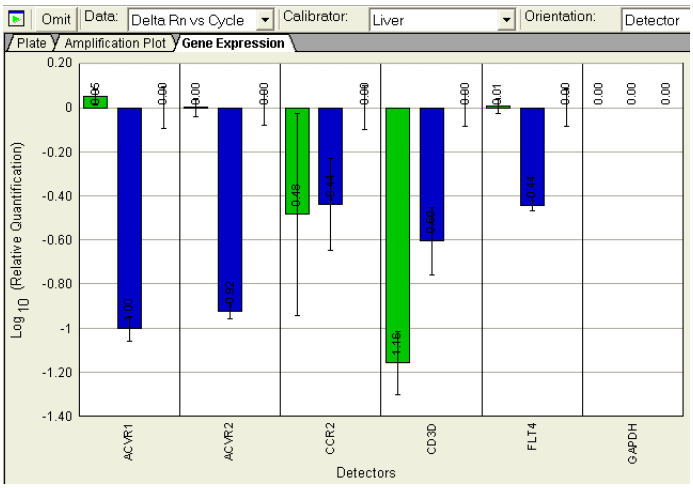
- Bar width
- 3D bars
- Autoscaling
- Data display as Log_{10} RQ or Raw RQ

Refer to the Online Help for more information about adjusting graph settings for gene expression plots.



Gene Expression Plot Orientation: Detector

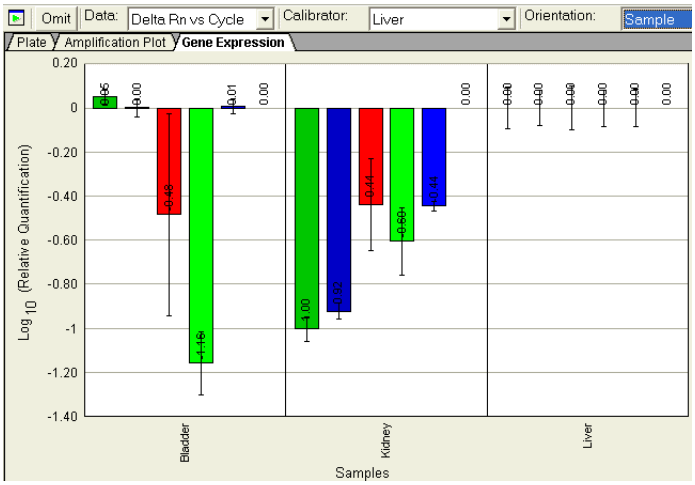
Detectors are plotted on the x-axis, and each bar shows the detector value of a single sample.



Notes

Gene Expression Plot Orientation: Sample

Samples are plotted on the x-axis, and each bar shows the set of sample values of a single detector.



Error Bars for Gene Expression Plots

The SDS software displays error bars for each column in the plot provided that the associated expression level was calculated from a group of two or more replicates. The error bars display the calculated maximum (RQMax) and minimum (RQMin) expression levels that represent standard error of the mean expression level (RQ value). Collectively, the upper and lower limits define the region of expression within which the true expression level value is likely to occur.

The SDS software calculates the error bars based on the RQMin/Max Confidence Level in the Analysis Settings dialog box (see page 38).

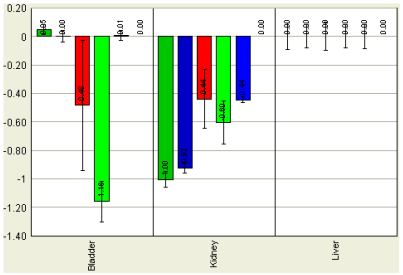
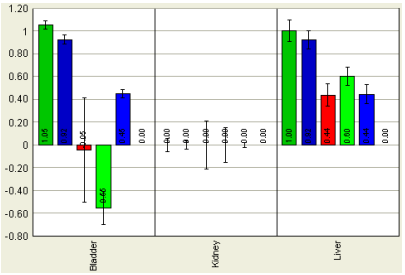
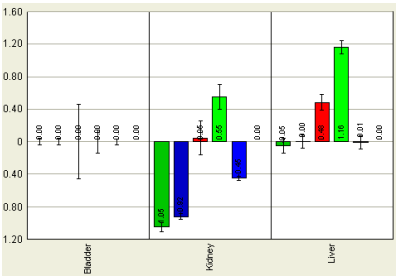
Notes

Reanalyzing an RQ Study

If you change any of the analysis settings, you must reanalyze the data before you can view results. (You can switch between the variations of the Amplification and Gene Expression plots without having to reanalyze the data.)

Suppose you select Liver as the calibrator, then perform an analysis. Next, you view the Amplification and Gene Expression plots. If you then want to use Kidney or Bladder as the calibrator, you need to reanalyze the data before viewing results.

Similarly, if you want to change the baseline or threshold values, the endogenous control, the control type, or the RQ Min/Max parameters, you need to reanalyze your data.

Calibrator	Gene Expression Plot
Liver	
Kidney	
Bladder	

Notes

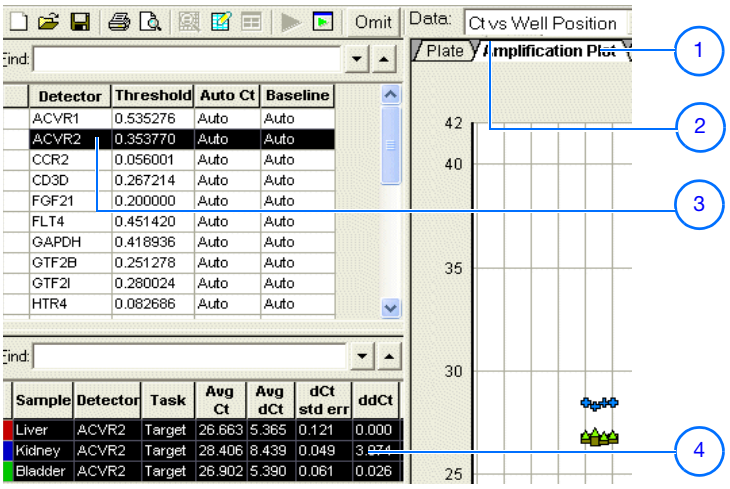
Omitting Samples from a Study

Experimental error may cause some wells to amplify insufficiently or not at all. These wells typically produce C_T values that differ significantly from the average for the associated replicate wells. If included in the calculations, these outlying wells (outliers) can result in erroneous measurements.

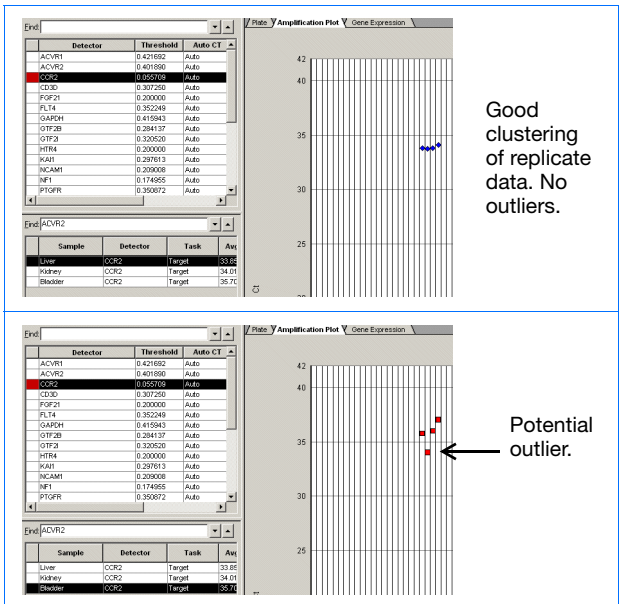
To ensure precise relative quantification, you must carefully view replicate groups for outliers. You can remove outliers manually using the C_T vs. Well Position Amplification Plot.

To remove samples from an RQ Study:

1. Select the **Amplification Plot** tab.
2. In the Data drop-down list, select **Ct vs. Well Position**.
3. In the RQ Detector grid, select a detector to examine. All samples that use this detector are displayed in the RQ Samples grid.
4. In the RQ Samples grid, click to select the samples to display in the Amplification Plot.


Omit
Data:
Ct vs Well Position
Plate
Amplification Plot
Detector | Threshold | Auto Ct | Baseline
ACVR1 | 0.535276 | Auto | Auto
ACVR2 | 0.353770 | Auto | Auto
CCR2 | 0.056001 | Auto | Auto
CD3D | 0.267214 | Auto | Auto
FGF21 | 0.200000 | Auto | Auto
FLT4 | 0.451420 | Auto | Auto
GAPDH | 0.418936 | Auto | Auto
GTF2B | 0.251278 | Auto | Auto
GTF2I | 0.280024 | Auto | Auto
HTR4 | 0.082686 | Auto | Auto
Sample | Detector | Task | Avg Ct | Avg dCt | dCt std err | ddCt
Liver | ACVR2 | Target | 26.663 | 5.365 | 0.121 | 0.000
Kidney | ACVR2 | Target | 28.406 | 8.439 | 0.049 | 3.074
Bladder | ACVR2 | Target | 26.902 | 5.390 | 0.061 | 0.026
42
40
35
30
25
1
2
3
4

5. Verify the uniformity of each replicate population by comparing the groupings of C_T values for the wells that make up the set.



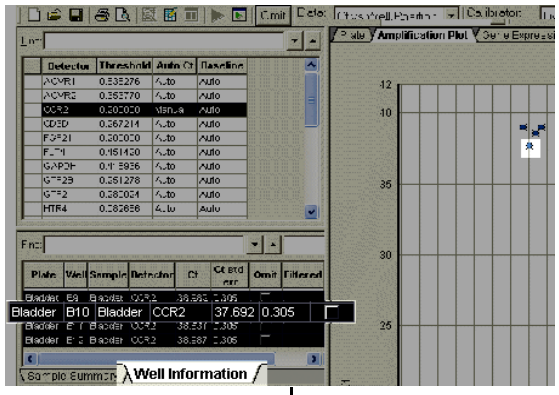
Notes

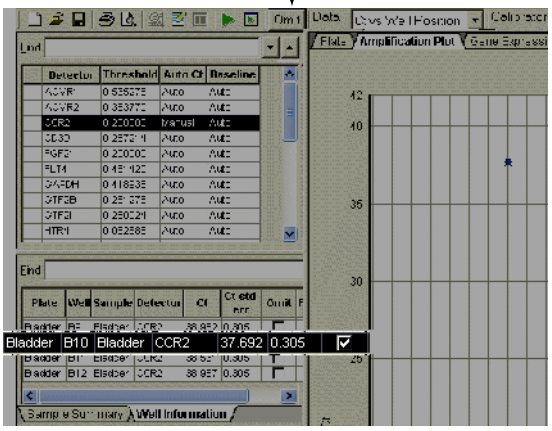
6. Do one of the following:
   - If outliers are present, select the **Well Information** tab, find the outlying sample, and select the **Omit** check box for the sample.
   - If outliers are not present, go to step 7.

7. Repeat steps 5 and 6 to screen the remaining replicate groups.

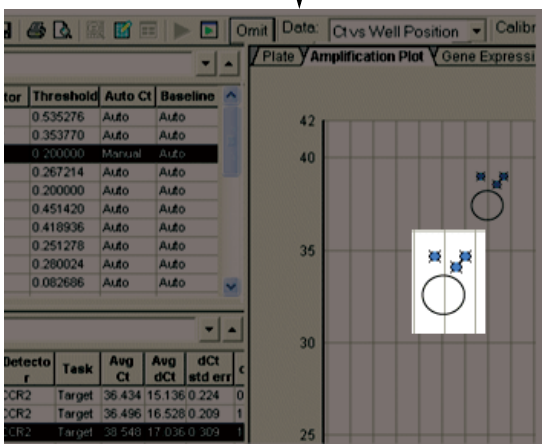
8. Select **Analysis > Analyze** (▶) to reanalyze the run without the outlying data.

9. Repeat steps 3 to 8 for other detectors you want to screen.





Select Omit.



The outlier is removed during analysis.

5

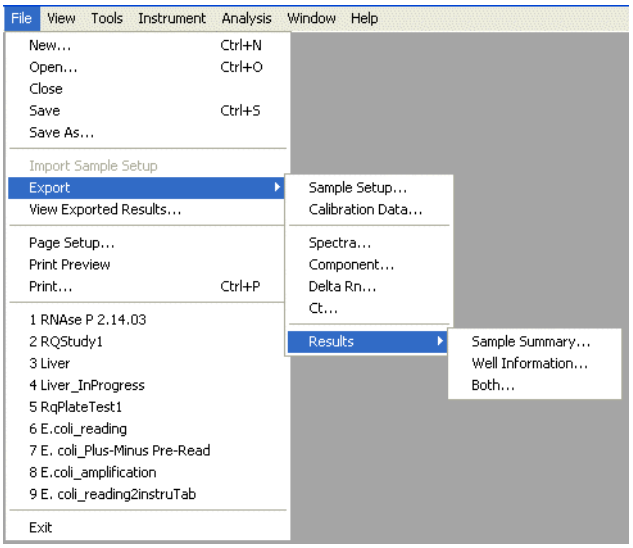
Notes

Exporting RQ Study Data

You can export numeric data from RQ studies into text files, which can then be imported into spreadsheet applications such as Excel.

1. Select **File** > **Export** > **Results**, then select the data type to export:
   - **Sample Summary** (*.csv)
   - **Well Information** (*.csv)
   - **Both** (*.csv)

   Refer to the Online Help for information about the export file types.



2. Enter a file name for the export file.

   Note: The name of the dialog box depends on the type of data you want to export.

3. Click **Save**.

Notes

Creating Detectors A

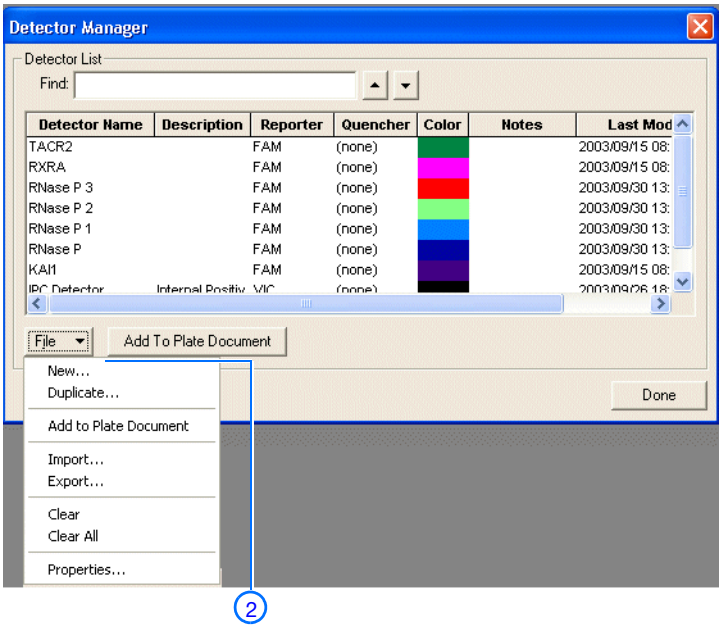
Before you can use a plate document to run a plate, you need to create and apply detectors for all samples on the plate. A detector is a virtual representation of a gene- or allele-specific nucleic acid probe reagent used for analyses performed on instruments.

To create a detector:

1. Select **Tools > Detector Manager**.

   Note: A plate document (any type) must be open before you can access the Tools menu.

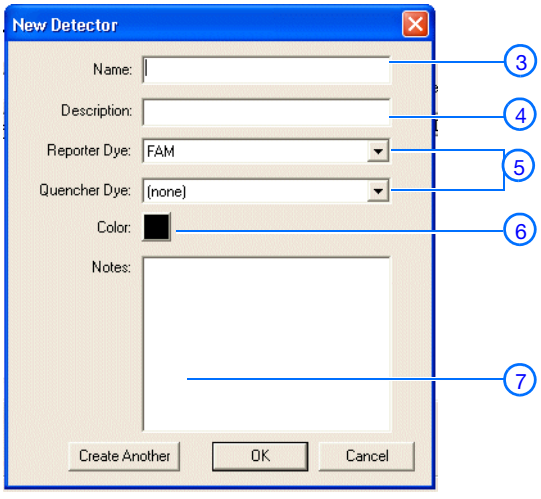
2. Select **File > New**.



3. In the New Detector dialog box, enter a name for the detector.

   IMPORTANT! The name of the detector must be unique and should reflect the target locus of the assay (such as GAPDH or RNase P). Do not use the same name for multiple detectors.

4. Optionally, click the **Description** field, then enter a brief description of the detector.



Notes

5. In the Reporter Dye and Quencher Dye drop-down lists, select the appropriate dyes for the detector.

   Note: The dyes that appear on the Reporter and Quencher Dye lists are those that have been previously entered using the Dye Manager. If the dye that you want to use does not appear in a list, use the Dye Manager to add the dye and then return to this step in this procedure. Refer to the Online Help for more information.

   Note: Select TAMRA as the quencher for TaqMan™ probes and None for TaqMan MGB probes.

6. Click the **Color** box, select a color to represent the detector using the Color dialog box, then click OK.

7. Optionally, click the **Notes** field, then enter any additional comments for the detector.

8. Click **OK** to save the detector and return to the Detector Manager.

9. Repeat steps 2 through 8 for the remaining detectors.

10. In the Detector Manager, click **Done** when you finish adding detectors.

Example Experiment

In the example RQ experiment, a detector is created for each target gene and the endogenous control. 24 detectors are created: 23 for the target genes and 1 for the endogenous control, GAPDH.

For example, the detector for the ACVR1 gene is named ACVR1 and assigned a yellow color. Because all Assays-on-Demand™ products have probes that are labeled with FAM™ dye, FAM was selected for the reporter dye. Additionally, Assays-on-Demand products use TaqMan MGB probes, which use nonfluorescent quenchers. No quencher dye is selected for the detector.

Note: Assays-on-Demand products are shipped with an assay information file (AIF). This text-based file contains information about the assays that you ordered, including the Applied Biosystems Assay ID number, well-location of each assay, primer concentration, and primer sequence. The file also indicates the reporter dyes and quenchers (if applicable) that are used for each assay. When creating detectors, you use the reporter dye and quencher information (and optionally, the gene name or symbol for the sample name). You can view the contents of AIFs in a spreadsheet program, such as Microsoft Excel.

Notes

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